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Award Number: W81XWH-13-2-0077

TITLE: Preventative Therapeutics for Heterotopic Ossification

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| October 2015 | Annual | 30 Sep 2014 - 29 Sep 2015 |

**4. TITLE AND SUBTITLE**

Preventative Therapeutics for Heterotopic Ossification

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-13-2-0077

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)**

The Geneva Foundation
917 Pacific Ave, Suite 600
Tacoma, WA 98402

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

In this project we study Heterotopic Ossification (HO) and are a potential novel therapy to cure it. HO consists of formation of extra bone within the muscles, near tendons and ligaments, inside the blood vessels and other places in the body. HO is triggered by trauma, burns, nerve damage, immobilization and other conditions and can also occur in patients undergoing large surgeries such as hip or knee replacement. Because trauma, burns and other severe wounds are regrettably common in our soldiers in the current war theaters and conflicts, HO often affects and afflicts many of them. The consequences of having HO are not minor. Patients with HO can experience loss of normal posture and movement, chronic pain, prosthesis fitting problems, formation of pressure ulcers, deep venous thrombosis and other health problems. Indeed, HO has emerged as the single most important barrier to functional activity and return-to-duty in a recent analysis of wounded active duty service- members. Subsequent infection remains one of the common and significant complications following blastrelated severe fracture and amputation with Acinetobacter Baumannii and Methicillin Resistant Staphylococcus Aureus (MRSA) being the most common isolate from combat wounds. To more precisely identify the cellular and molecular changes associated trauma-induced HO formation and to test the potential in vivo inhibitor effects of an retinoic acid receptor- agonist called palovarotene, we will use an established rat model of combat-related extremity injury/amputation that incorporates the critical elements commonly associated with combat injury namely blast injury, femur fracture and amputations, soft tissue injury and bioburden.

**15. SUBJECT TERMS**

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 9 | 19b. TELEPHONE NUMBER *(include area code)* |

**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

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# INTRODUCTION

In this project we study Heterotopic Ossification (HO) and are a potential novel therapy to cure it. HO consists of formation of extra bone within the muscles, near tendons and ligaments, inside the blood vessels and other places in the body. HO is triggered by trauma, burns, nerve damage, immobilization and other conditions and can also occur in patients undergoing large surgeries such as hip or knee replacement. Because trauma, burns and other severe wounds are regrettably common in our soldiers in the current war theaters and conflicts, HO often affects and afflicts many of them. The consequences of having HO are not minor. Patients with HO can experience loss of normal posture and movement, chronic pain, prosthesis fitting problems, formation of pressure ulcers, deep venous thrombosis and other health problems. Indeed, HO has emerged as the single most important barrier to functional activity and return-to-duty in a recent analysis of wounded active duty service- members. Subsequent infection remains one of the common and significant complications following blast-related severe fracture and amputation with *Acinetobacter Baumannii* and *Methicillin Resistant Staphylococcus Aureus* (MRSA) being the most common isolate from combat wounds. To more precisely identify the cellular and molecular changes associated trauma-induced HO formation and to test the potential in vivo inhibitor effects of an retinoic acid receptor-$\gamma$ agonist called palovarotene, we will use an established rat model of combat-related extremity injury/amputation that incorporates the critical elements commonly associated with combat injury namely blast injury, femur fracture and amputations, soft tissue injury and bioburden.


### KEYWORDS

heterotopic ossification, traumatic injury, ectopic bone, palovarotene, amputation, combat wounds, crush injury, bioburden, osteogenesis, chondrogenesis, gene expression, extremity injuries, and blast overpressure exposure.


# OVERALL PROJECT SUMMARY

### NMRC-Regenerative Medicine Department Role (SOW) in the Partnership Award

**Proposal Specific Aim 3**: To determine whether the retinoid agonists block blast- and combat-related HO (months 1-36):

*Task 3a. Implement the rat blast-injury model to include bacterial infection and HO (**Completed July 2015**).*
Current objective: To determine the effects of *Acinetobacter Baumannii* and *Methicillin Resistant Staphylococcus Aureus* (MRSA) infection on the rate development and severity of HO formation.
Results, Progress and Accomplishments with Discussion:

We have completed a series of experiments to determine if (1) the presence of bioburden (*Acinetobacter baumannii* and *methicillin-resistant Staphylococcus aureus*

[MRSA]) increases the magnitude of ectopic bone formation in traumatized muscle after amputation; and (2) what persistent effects bacterial contamination has on late microbial flora within the amputation site.

Using a blast-related HO model, we exposed 48 rats to blast overpressure, femur fracture, crush injury, and subsequent immediate transfemoral amputation through the zone of injury. Control injured rats (n=8) were inoculated beneath the myodesis with phosphate-buffered saline (PBS) not containing bacteria (vehicle) and treatment rats were inoculated with 1 x $10^6$ colony-forming units of A. baumannii (n=20) or MRSA (n=20). All animals formed HO. Heterotopic ossification was determined by quantitative volumetric measurements of ectopic bone on the 12-week post injury using microCT imaging and qualitative histomorphometry for assessment of new bone formation in the residual limb. Bone marrow and muscle tissue biopsies were collected from the residual limb at 12-weeks to quantitatively measure the bioburden load and to qualitatively determine the species-level identification of the bacterial flora.

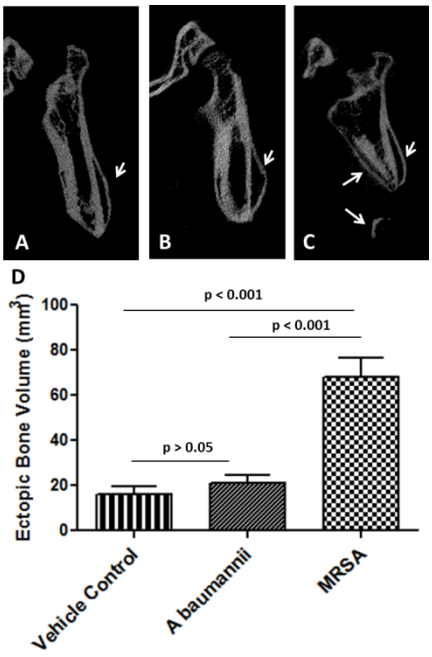



**Figure 1**. Representative longitudinal 12-week micro-CT images of the residual femurs of rats inoculated with (A) Vehicle control (PBS; noninfected control); (B) *A baumannii;* and (C) MRSA are shown. The arrow highlights the varying degree of ectopic bone formed as a results of treatment. (D) The amount of ectopic bone was quantified 12 weeks postinjury

At 12 weeks, 100% of the injured rats developed radiographic evidence of HO. No radiographic evidence of neurogenic HO development (around joints and/or in the soft tissue distant from the fracture/amputation site) in our model or in blast only treated rats. We measured a greater volume of HO in rats infected with MRSA (68.9 ± 8.6 $mm^3$; 95% CI, 50.52 - 85.55) when compared with *A. baumannii* (20.9 ± 3.7 $mm^3$; 95% CI, 13.61 - 28.14; $p < 0.001$) or vehicle (16.3 ± 3.2 $mm^3$; 95% CI; 10.06 - 22.47; $p < 0.001$) (**Figure 1**). Soft tissue and marrow from the residual limb of rats inoculated with *A. baumannii* tested negative for *A. baumannii infection* but were positive for other strains of bacteria (1.33 x $10^2$ ± 0.89 x $10^2$; 95% CI, -0.42 x $10^2$ - 3.08 x $10^2$ and 1.25 x $10^6$ ± 0.69 x $10^6$; 95% CI, -0.13 x $10^6$ – 2.60 x $10^6$ colony-forming units in bone marrow and muscle tissue respectively), whereas tissue from MRSA-infected rats

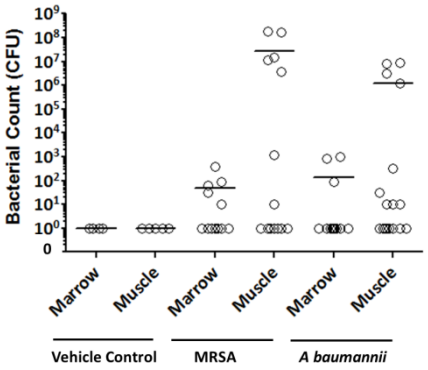



**Figure 2**. Bacterial counts [in colony forming units (CFUs) converted to log scale] in the marrow compartment and soft tissue of rats infected with vehicle control (PBS; noninfected control), MRSA, and A. baumanni after 12 weeks Each data point represents the actual CFU value for each animal in each treatment group while the horizontal bar indicates the mean CFU for each treatment group.

contained MRSA only ($4.84 \times 10^1 \pm 3.22 \times 10^1$; 95% CI, $-1.47 \times 10^1 - 11.1 \times 10^1$ and $2.80 \times 10^7 \pm 1.73 \times 10^7$; 95% CI, $-0.60 \times 10^7 - 6.20 \times 10^7$ in bone marrow and muscle tissue respectively) (**Figure 2**). All rats inoculated with MRSA tested positive for MRSA, whereas rats inoculated with *A. baumannii* tested positive for other microorganisms as detailed in **Table 1**.

**Table 1. List of bacteria present in the marrow compartment and soft tissue 12 weeks post injury.**

| | **Vehicle control** | ***Acinetobacter baumannii*** | **Methicillin Resistant *Staphylococcus aureus* (MRSA)** |
|---|---|---|---|
| **Marrow** | Negative | *Arcanobacterium haemolyticum*; *Enterobacter cloacae* and *Enterococcus faecalis* | *Staphylococcus aureus* |
| **Muscle Tissue** | Negative | *Arcanobacterium haemolyticum, Streptococcus porcinus, Staphylococcus cohnii ssp urealyticum, Staphylococcus xylosus, Gardnerella vaginalis, Pasteurella multocida, Enterobacter cloacae* and *Enterococcus faecalis* | *Staphylococcus aureus* |

Studies evaluating the effects of polymicrobial infection ($1 \times 10^6$ MRSA plus $1 \times 10^6$ *A. baumannii*) on HO formation showed no augmentation in ectopic bone formation above that observed with MRSA alone.

Our findings demonstrate that persistent infection with MRSA results in a greater volume of ectopic bone formation, which may be the result of chronic soft tissue inflammation, and that early wound colonization may be a key risk factor. In summary, interventions that mitigate wound contamination and inflammation (such as early débridement, systemic and local antibiotics) may also have a beneficial effect with regard to the mitigation of HO formation, and should be evaluated with that potential in mind in future preclinical studies.

Studies under Task 3a have been completed. Results have been complied, analyzed and presented by our team members at several international orthopaedic scientific conferences dealing with acute extremity injury care. In addition, these critical findings

were published to a peer-reviewed publication in the June 2015 issue of Clinical Orthopaedic Related Research (CORR). The referenced published manuscript is attached for reference.

*Task 3b. Test drug effectiveness, regimens and systemic versus local delivery (months 8-30).*

Current objective: In order to optimize the timing of drug-based prophylactic intervention following blast-related combat injury, it is important to define the (1) early histological changes in soft tissue architecture, vascularity, collagen deposition and cartilage development and their correlation with later ectopic bone development following blast-related traumatic injury; (2) the effects of nuclear retinoic acid receptor-γ agonist (RARAγ; palovarotene) has on ectopic endochondral bone formation our blast-related extremity injury HO model in rats; (3) and the expression profile of early chondrogenic and osteogenic gene transcripts in traumatized tissues collected from untreated and RARAγ-treated rats.

Results, Progress and Accomplishments with Discussion:

Defining the early development phase of HO in relationship to concurrent wound healing is critical to selection of candidate means of prophylaxis and, importantly, the timing of their administration after high-energy extremity injuries. Studies under Task3b-subtask 1 which focused on assessing the early histological changes in soft tissue architecture, vascularity, collagen deposition and cartilage development and their correlation with later ectopic bone development following blast-related traumatic injury have been completed.

Using the physiologic model of combat-related HO described above evaluated (1) the timing of early chondrogenesis, cartilage formation and radiographic ectopic bone development; and (2) the early cartilage and bone-related gene and protein patterns in traumatized soft tissue subsequent to calcium deposition, tissue mineralization, and ectopic bone formation. Cohorts of four to eight rats per time point were euthanized on postinjury days 3, 5, 7, 10, 14, 21, and 28, to detect and visualize histologically the early stages of the HO disease process, whereas a cohort of four sham-treated (neither blasted nor injured) naïve rats euthanized on day-3 served as controls. The residual injured and contralateral femurs with attached associated muscle tissue were surgically removed and placed in 10% formalin. The femurs were decalcified in 5% formic acid, embedded in paraffin, longitudinal sectioned (5 μm), and stained with hematoxylin-eosin (Histoserv, Inc, Germantown, MD, USA). Qualitative observation of wound healing and early ectopic endochondroal bone development (mesenchymal condensation, chondrocyte differentiation, chondrogenesis, hypertrophic vascularized cartilage, hyaline cartilage development, extracellular maturation, and soft-tisse mineralization). Histologic assessment was performed by a pathologist who was blinded to the study. Foci of proliferative/hypertrophic chondrocytes were observed in tissue surrounding the amputation site (**Figure 3, panels A-F**) as early as 5 days and certainly by postoperative day 10. By day 14, endochondral ossification was evident as the ectopic chondrocyte-rich basophilic hyaline cartilage was replaced with acidophilic

bone matrix (osteoid) later followed by the immature woven bone typical of HO arising from the process of endochondral ossification (**Figures 3, panels G-I**). None of the contralateral limbs from blast-injured rats or limbs from sham-treated rats developed radiographic or histologic evidence of HO.

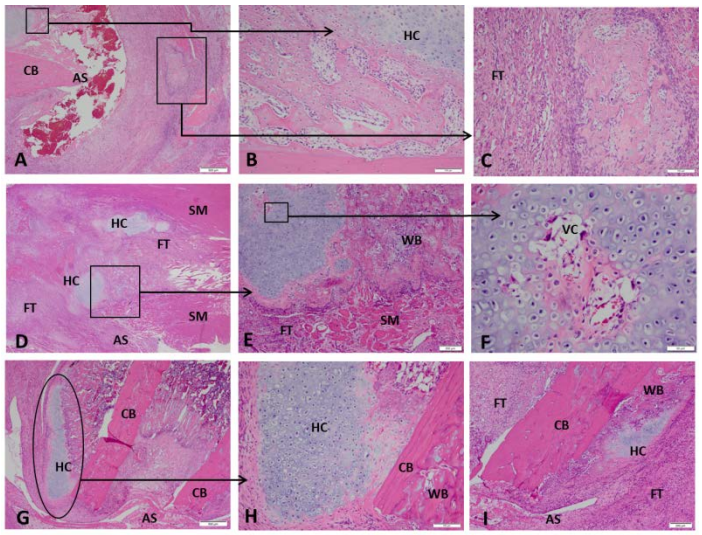



**Figure 3**. Histological evidence of early HO formation at the site of amputation postinjury day 7 (A-C), day 10 (D-F), and day 14 (G-I) (Stain, Hematoxylin and eosin). For detailed evaluation, higher magnification images of five selected regions are shown. Foci of hyaline and vascularized cartilage with woven bone are observed in the soft tissue surrounding the site of amputation at postinjury day 10-14. AS = amputation site; CB = cortical bone; FT = fibroblastic tissue; HC = hyaline cartilage; SM = skeletal muscle; VC = vascularized cartilage; WB = woven bone.

To evaluate early condrogenic, oasteogenic and angiogenic cell signaling post injury, small tissue biopsies of tissue surrounding the amputation site were immediately placed in either RNAlaterTM (Ambion Inc, Austin, TX, USA) at 4 °C for 48 hours or snap-frozen in dry ice for gene expression and protein analysis, respectively. Total RNA was isolated from skeletal muscle samples as using standard procedures. A custom-made low-density reverse transcription-polymerase chain reaction (RT-PCR) array consisting of 96 primer sets (including respective forward and reverse primers) for 83 rat-specific osteogenic, chondrogenic, adipogenic, and angiogenic genes as well as six housekeeping and seven quality control genes (SABiosciences, Gaithersburg, MD, USA) was used to assess gene expression (genes and their function listed in Quantitative RT-PCR was conducted. Cycle threshold (Ct) measurements per samples were normalized using GAPDH. Relative expression between sham-treated naïve rats

| | Days Postinjury | | | | | | |
|---|---|---|---|---|---|---|---|
| Gene Expression | 3 | 5 | 7 | 10 | 14 | 21 | 28 |
| ADIPOQ | | | | p=0.029 | p=0.029 | | |
| OCN | p=0.032 | | | | | | |
| CCL2 | | | p=0.016 | p=0.029 | | | |
| CCL3 | p=0.016 | | p=0.016 | p=0.029 | | | |
| COL1α1 | | | p=0.016 | | | | |
| CSF3 | p=0.032 | | | | | | |
| CXCL5 | p=0.016 | | | | | | |
| FGF10 | | | p=0.032 | | | | |
| GUSB | | | p=0.032 | p=0.029 | | | |
| HAS1 | | p=0.038 | p=0.016 | p=0.029 | | | |
| ILBS | | | | | | | |
| IGF2 | | | p=0.016 | p=0.029 | p=0.029 | | |
| IL-1β | | | | p=0.029 | | | |
| IL-6 | p=0.016 | | p=0.016 | | | | |
| ITGAX | | | p=0.016 | p=0.029 | | | |
| MMP9 | p=0.032 | p=0.038 | | | | | |
| PHEX | p=0.016 | | | p=0.029 | p=0.029 | | |
| POU5F1 | p=0.016 | | | | | | |
| PPARG | | | | p=0.029 | p=0.029 | | |
| RUNX2 | | | p=0.032 | p=0.029 | | | |
| SCARB1 | | | p=0.016 | p=0.029 | p=0.029 | | |

A. INJURED LIMB

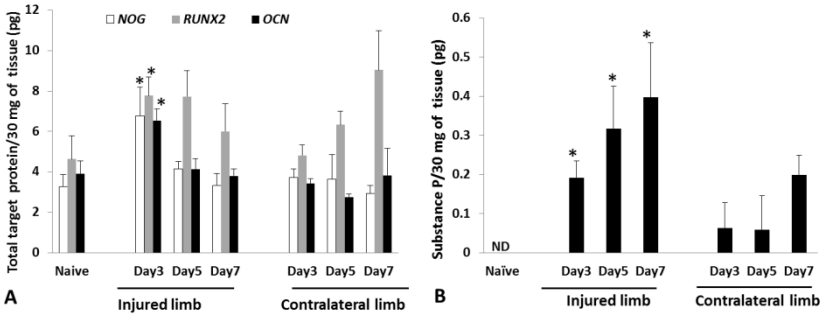
| | Days Postinjury | | | | | | |
|---|---|---|---|---|---|---|---|
| Gene Expression | 3 | 5 | 7 | 10 | 14 | 21 | 28 |
| ADIPOQ | | | | | | | |
| OCN | | p=0.024 | | | | | |
| CCL2 | | | | | | | |
| CCL3 | | p=0.024 | | | | | |
| COL1α1 | | | | | | | |
| CSF3 | | | | | | | |
| CXCL5 | | | | | | | |
| FGF10 | | | | | | | |
| GUSB | | | | | | | |
| HAS1 | | | p=0.032 | | | | |
| ILBS | P=0.042 | p=0.024 | | | | | |
| IGF2 | | | p=0.016 | | p=0.029 | | |
| IL-1β | | p=0.006 | | | | | |
| IL-6 | | p=0.024 | | | | | |
| ITGAX | | | | | | | |
| MMP9 | | p=0.042 | | | | | |
| PHEX | | | | | | | |
| POU5F1 | p=0.042 | | | | | | |
| PPARG | | | | | | | |
| RUNX2 | | | | | | | |
| SCARB1 | | | | | | | |

B. CONTRALATERAL LIMB

| Fold Change |
|---|
| 0-3 |
| 3-5 |
| 5-10 |
| 10-100 |
| 100-1000 |

**Figure 4**. A transcript heat map depicting the expression level of the subset of the 83 rat chondrogenic, osteogenic, and angiogenic related gene targets whose differential expression was greater than threefold compared with the expression level in sham-treated control muscle. (A) Contralateral leg and (B) injured leg and significantly different ($p < 0.05$; Mann-Whitney U-test) compared with sham-treated rats are noted with asterisk.

and the injured limb muscle samples was determined using the comparative Ct method ($2^{-\Delta\Delta Ct}$). In comparison to sham-treated naïve rats, genes that were deferentially expressed at least threefold were depicted using a heat map. Assays with Ct values greater than 35 cycles were considered not expressed. As shown in Figure 4, genes involved in chondrogenesis, osteogenesis, wound healing/tissue repair, and adipogenesis were notably overexpressed (greater than threefold) at the amputation site, whereas all angiogenic targets were unchanged (less than threefold) in comparison to quadriceps muscle tissue collected from the contralateral limb and sham-treated naïve rats. The in vivo tissue production of key osteogenic proteins (NOG $p = 0.04$), OCN ($p = 0.023$), and RUNX-2 ($p = 0.04$) was elevated at 3 days post injury relative to normal muscle tissue collected from sham-treated naïve controls (**Figure 5A**). In addition, we observed that the amount of the peripherally released neurotransmitter substance P (SP-1) was higher in the injured limb at 3 to 7 days postinjury (0.2 ± 0.04 ng, 0.32 ± 0.10 ng, and 0.4 ± 0.13 ng/per 30 mg of tissue) when compared with sham-treated naïve control muscle ($p = 0.002$, $p = 0.009$, and $p = 0.01$, respectively) and muscle collected from the contralateral leg, which was subjected only to blast-related trauma (**Figure 5B**). There were no differences observed in concentrations of neurokinin A, CGRP, or BMP-2 (data not shown).




C

| | Mean | Standard Deviation | 95 % CI | P value |
|---|---|---|---|---|
| NOG - Naive | 3.25 | 1.04 | 2.07-4.43 | |
| NOG - Day3 Injured Limb | 6.78 | 2.40 | 4.06-9.50 | 0.04 |
| | | | | |
| OCN - Naive | 3.89 | 1.30 | 2.61-5.17 | |
| OCN - Day3 Injured Limb | 6.54 | 0.80 | 5.43-7.65 | 0.02 |
| | | | | |
| RUNX2 - Naive | 4.63 | 1.99 | 2.37-6.89 | |
| RUNX2 - Day3 Injured Limb | 7.76 | 1.33 | 5.92-9.61 | 0.04 |
| | | | | |
| SP-1 - Naive | ND | ND | ND | |
| SP-1 - Day3 Injured Limb | 0.19 | 0.08 | 0.10-0.27 | 0.002 |
| SP-1 - Day5 Injured Limb | 0.31 | 0.21 | 0.10-0.53 | 0.009 |
| SP-1 - Day7 Injured Limb | 0.39 | 0.27 | 0.12-0.67 | 0.01 |

**Figure 5**. Protein quantitation of NOG, OCN, and RUNX-2 (A) and SP-1 (B). The levels of NOG, OCN, and RUNX-2 are statistically significant from the sham-treated (naïve) rats at 3 days postinjury, whereas the levels of SP-1 are significantly different from the sham-treated (naïve) rats at 3-7 days postinjury (*p <0.05; Student's t-test).

Results have been complied, analyzed and presented by our team members at several international orthopaedic scientific conferences dealing with acute extremity injury care. In addition, these pivotal findings were published in the June 2015 issue of Clinical Orthopaedic Related Research (CORR). The referenced published manuscript is attached for reference.

For Tasks3b-subtask 2&3, we have completed a comprehensive set of studies evaluating the in vivo effects of palovarotene (RARA$\gamma$), which targets early chondrogenesis (cartilage formation), on ectopic bone formation in our described model

for traumatic and blast-related HO. We exposed 104 adult male Sprague-Dawley rats to blast overpressure, femur fracture, quadriceps crush injury, and transfemoral amputation through zone of injury, followed by bacterial inoculation with an MRSA strain isolated from combat wounds. Our prior work using this model demonstrated early histologic evidence of chondrogenic differentiation between 3-10 days post injury, thereby suggesting the optimal treatment window for prophylactic agents, such as RARAγ. Cohorts of rats were given either RARAγ or vehicle by enteral gavage, beginning on either post-operative day (POD) 1 or 5. Rats were monitored for 12 weeks for evidence of wound dehiscence and subsequently underwent sacrifice and quantification of volumetric ectopic bone attenuation using microCT imaging (**Figures 6 and 7**). Amongst rats infected with MRSA, those treated with control vehicle on POD-1 and POD-5 developed a mean ectopic bone volume of 43.3 ± 10.2 $mm^3$ and 38.9 ± 8.5 $mm^3$ respectively, compared to rats that received RARAγ on POD-1 (16.4 ± 3.9 $mm^3$; p=0.01) and POD-5 (16 ± 6.1 $mm^3$; p=0.04).

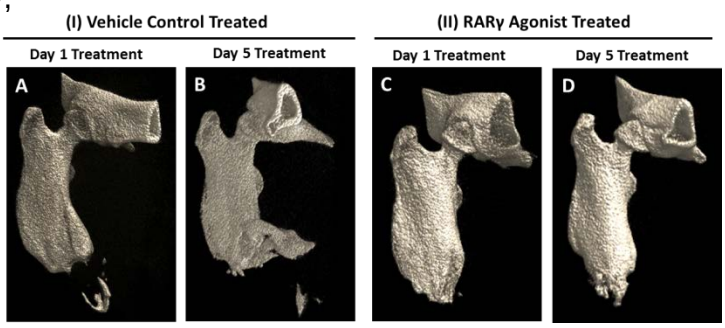



**Figure 6**. Representative longitudinal 12-week micro-CT reconstructed images of residual femurs of rats treated with (A-B) vehicle control (corn oil/DMSO) and (C-D) RAR-γ agonist administered via enternal gavage starting at either POD-1 or POD-5

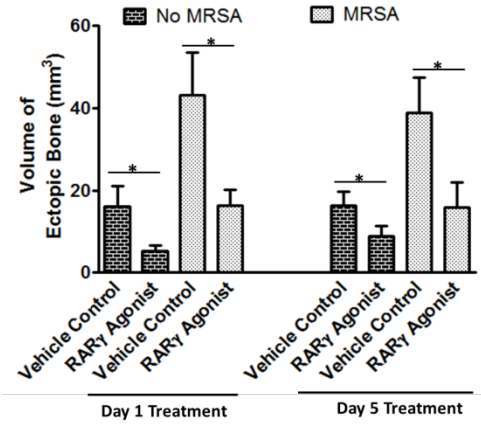



**Figure 7**. Graphical representation of the amount of ectopic bone, quantified using 12 weeks µ-CT images, of rats treated with vehicle control and RAR-γ agonist at POD-1 and POD-1 with and without MRSA (*= p <0.05 using the Welch's two Sample t-test)

From tissue collected around the amputation site in vehicle-treated rats, hematoxylin eosin staining demonstrated endochondral ossification in the form of foci of chondroblast progenitor cell proliferation and differentiation, hypertrophic chondrocytes, vascularized hyaline cartilage, acidophilic bone matrix (osteoid), and immature woven bone at 15-28 days post injury with the development of an endosteal callus ( " bony bridge') across the medullary cavity at the amputation site (**Figure 8, panels A-D**). By 12 weeks, areas of inflammation with foci of intense bacterial multiplication and necrosis were noted in the soft tissue wherein ectopic bone areas, containing empty lacunae, often became walled off by fibrous tissue to form a necrotizing pyogranuloma leading to ectopic bone resorption/remodeling (**Figure 8, panels E-F**). Comparatively, in the RAR-γ agonist treated rats, we observed fewer foci of active chondrogenesis and vascularized hyaline cartilage but rather extensive areas filled with fibrous connective tissue at 15 days post injury (**Figure 8, panels G-H**). Histological investigation showed

that the medullary cavity was largely filled/closed with fibrous connective tissue after 3-4 weeks post injury (**Figure 8, panels I-L**). Histological analysis of residual limbs treated with RARAγ demonstrated dampened and altered endochondral ossification patterns; developing chondrocytes were later replaced by fibroblastic tissue, a finding corroborated by detected marked upregulation of gene products vital to cartilage catabolism (MMP9) and fibroblastic growth factor (FGF1 and FGF10) (data not shown). Therefore, early post-injury RARAγ administration represents a promising prophylactic therapy in combat wounded patients at risk for developing heterotopic ossification.

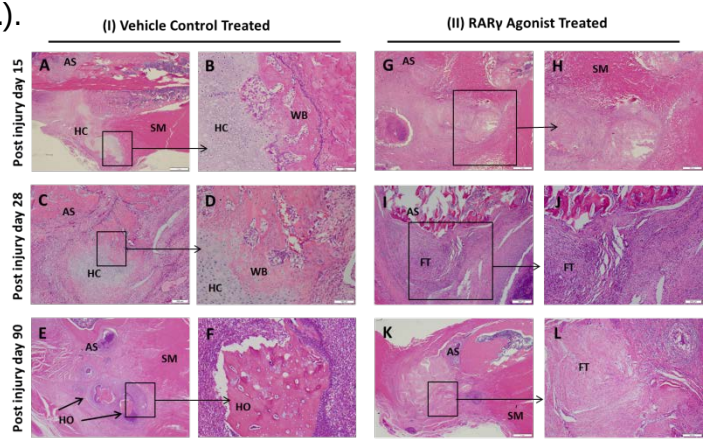



**Figure 8**. Histological (hematoxylin and eosin; H&E) progression of HO formation in vehicle control treated rats at 15 days (A-B), 28 days (C-D) and 90 days (E-F) post injury followed by marked alteration of chondrogeneis in the RAR-γ agonist treated rats at 15 days post injury (G-H), leading to fibroblastic replacement at 28 days (I-J) and clinically significant attenuation of ectopic bone at 90 days (K-L). AS = amputation site; FT = fibroblastic tissue; HC = hyaline cartilage; SM = skeletal muscle; WB = woven bone and HO = heterotopic ossification

Results have been complied, analyzed and presented by our team members at several international orthopaedic scientific conferences dealing with acute extremity injury care. In addition, we are in the final revision/review phase of a manuscript reporting on these important and insightful findings. Targeted date for submission is February, 2015 to the peer-reviewed orthopaedic journal BONE.

*Task 3c. Analyze wound healing and muscle repair (months 18-36).*

We have completed a series of in-depth experimentation to evaluate the short-term and/or long-term and effects of therapeutic intervention with RARAγ (palovarotene) on delayed wound healing and wound dehiscence following blast-related traumatic injury (**Figure 9**). We observed no statistical difference in the number of wounds that dehisced between vehicle control treated (-MRSA) on POD-1 and POD-5 (2/6; 33%) and (1/8; 13%) respectively, compared to rats treated with RARAγ (2/8; 25%) and (0/8; 0%). Vehicle control treated (+MRSA) rats showed increased but statistically insignificant number of delayed dehisced wounds on POD-1 and POD-5 (1/5; 20%) vs. (2/5; 40%) respectively, compared to rats treated with RARAγ (5/8; 63%) vs. (5/8; 63%). The number of wound débridements were not

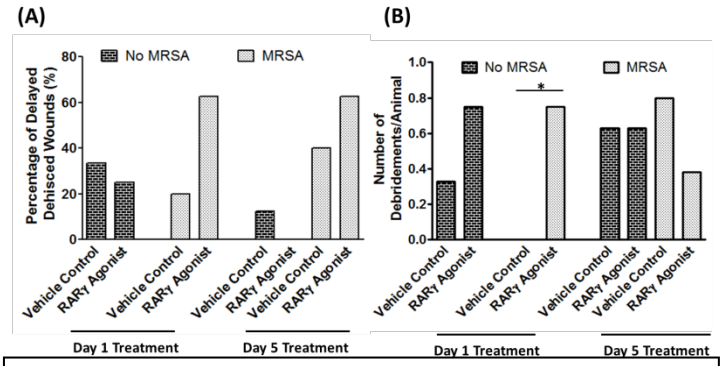



**Figure 9**. Effect of RAR-γ agonist on (A) delayed wound healing, demonstrated by percentage of wounds that dehisced after 21 days of initial wound closure and (B) the normalized number of debridements per experimental group (*= p <0.05 using the Fisher test).

significant except for when treated with RARAγ at POD-1 in the presence of MRSA (6/8; 0.75) compared to vehicle control treated (0/8; 0.0; p=0.001). Results from these studies indicate that doses of RARAγ (palovarotene) that significantly attenuate development of ectopic bone in a severe extremity trauma model did not have untenable deleterious effects on the healing process of large and complex wounds.

## KEY RESEARCH ACCOMPLISHMENTS

- We have defined the early cellular and molecular signaling development phases of HO development in our blast-related HO model.
- We have defined the therapeutic window for targeting early chondrogenesis, vasculogeneis and osteogenesis to inhibit the synthesis of cartilage and early ectopic bone formation without adverse effects on physiologic early wound healing processes such as tissue revascularization and granulation tissue development.
- We have characterized the effects of bioburden, specifically *Acinetobacter Baumannii* and *Methicillin Resistant Staphylococcus Aureus* (MRSA) infection, on the rate development and severity of ectopic bone formation (HO).
- We have demonstrated that the administration of palovarotene significantly attenuates ectopic bone formation, without major effects on wound closure/healing, when administered orally every other day for 2-weeks when administered shortly after blast-related polytraumatic extremity injury (1-5-days).
- We have published two pivotal peer-reviewed manuscripts encompassing our highly productive accomplishments over the last 2 years. A third manuscript is under preparation targeted for submission to the journal *BONE*. In addition, we have presented these research findings at multiple international scientific forums.

### CONCLUSION

The critical objectives and milestones for each specific task of Aim 3 of the partnership grant have been completed.

- The team is now poised for publishing an additional manuscript reporting the effects of RARAγ on attenuating ectopic bone formation following blast-related polytraumatic injury. Our goal is to submit to the journal of BONE within the 1$^{st}$ quarter of year 3.
- In year 3, we will complete an additional series of comprehensive experiments aimed at testing a higher dose of RARAγ (palovarotene) with treatment adminsitered on daily basis for the first 14-days post injury.

## PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

### Lay Press
Nothing to report.

### Peer-Reviewed Scientific Journals
1. Qureshi AT, Crump EK, Pavey GJ, Hope DN, Forsberg, JA and Davis TA. 2015. Early Characterization of Blast-Related Heterotopic Ossification in a Rat Model. Clinical Orthopaedics and Related Research 473:2831-2839.
2. Pavey GJ, Qureshi AT, Hope DN, Pavlicek RL, Potter BK, Forsberg JA and Davis TA. 2015. Bioburden Increases Heterotopic Ossification Formation in an Established Rat Model. Clinical Orthopaedics and Related Research 473:2840-2847.

### Invited Articles
Nothing to report.

### Abstracts
1. Gabriel J. Pavey, Ammar T. Qureshi, Allison Tomasino, Danett Bishop, Maurizio Pacifici, Masahiro Iwamoto, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Targeted stimulation of retinoic acid receptor signaling mitigates the formation of heterotopic ossification in an established blast-related traumatic injury model. Orthopedic Trauma Association (OTA) 2015. Oral Presentation

3. Ammar T. Qureshi, Erica Crump, Donald Hope, Gabriel J. Pavey, Jonathan A. Forsberg and Thomas A. Davis. Early Histological and Molecular Characterization of the Local Tissue Microenvironment Following Blast-Related Post-Traumatic Injury in a Rat Model of Heterotopic Ossification. Poster- Military Health System Research Symposium (MHSRS) 2015. Poster Presentation.

4. Gabriel J. Pavey, Ammar T. Qureshi, Allison Tomasino, Danett Bishop, Maurizio Pacifici, Mashahiro Iwamoto, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Targeted stimulation of retinoic acid receptor signaling mitigates the formation of heterotopic ossification in an established blast-related traumatic injury model. Oral Presentation- Military Health System Research Symposium (MHSRS) 2015. Oral Presentation.

5. Gabriel J. Pavey, Ammar T. Qureshi; Donald N. Hope, Rebecca L. Pavlicek, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Evaluation of Bioburden on the Development of Heterotopic Ossification in an Established Rat Model. Military Health System Research Symposium (MHSRS) 2015. Poster Presentation.

6. Ammar T. Qureshi, Gabriel J. Pavey, Donald N. Hope, Rebecca L. Pavlicek, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Evaluation of Bioburden on the Development of Heterotopic Ossification in an Established Rat Model. Southern Orthopedic Association 2015. Oral Presentation.

7. Gabriel J. Pavey, Ammar T. Qureshi, Allison Tomasino, Danett Bishop, Maurizio Pacifici, Mashahiro Iwamoto, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Targeted stimulation of retinoic acid receptor signaling mitigates the formation of heterotopic ossification in an established blast-related traumatic injury model. Walter Reed Research Day 2015. Oral Presentation.

8. Gabriel J. Pavey, Ammar T. Qureshi, Allison Tomasino, Danett Bishop, Maurizio Pacifici, Mashahiro Iwamoto, Benjamin K. Potter, Thomas A. Davis, Jonathan A. Forsberg. Targeted stimulation of retinoic acid receptor signaling mitigates the formation of heterotopic ossification in an established blast-related traumatic injury model. Walter Reed Research Day 2015- Poster Presentation.

**Presentations**
See abstract section above for oral and poster presentations.

## INVENTIONS, PATENTS AND LICENSES

Nothing to report.

## REPORTABLE OUTCOMES

Nothing to report.

## OTHER ACHIEVEMENTS

Nothing to report.

## REFERENCES

No references cited

## APPENDICES

1. Quad Chart

Clin Orthop Relat Res (2015) 473:2840–2847
DOI 10.1007/s11999-015-4272-3

Clinical Orthopaedics and Related Research®
A Publication of The Association of Bone and Joint Surgeons®



SYMPOSIUM: RESEARCH ADVANCES AFTER A DECADE OF WAR

# Bioburden Increases Heterotopic Ossification Formation in an Established Rat Model

**Gabriel J. Pavey MD, Ammar T. Qureshi PhD, Donald N. Hope MD, Rebecca L. Pavlicek PhD, Benjamin K. Potter MD, Jonathan A. Forsberg MD, Thomas A. Davis PhD**

Published online: 31 March 2015


**Abstract**

*Background* Heterotopic ossification (HO) develops in a majority of combat-related amputations wherein early bacterial colonization has been considered a potential early risk factor. Our group has recently developed a small animal model of trauma-induced HO that incorporates many of the multifaceted injury patterns of combat trauma in the absence of bacterial contamination and subsequent wound colonization.

*Questions/purposes* We sought to determine if (1) the presence of bioburden (*Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* [MRSA]) increases the magnitude of ectopic bone formation in traumatized muscle after amputation; and (2) what persistent effects bacterial contamination has on late microbial flora within the amputation site.

*Methods* Using a blast-related HO model, we exposed 48 rats to blast overpressure, femur fracture, crush injury, and subsequent immediate transfemoral amputation through the zone of injury. Control injured rats (n = 8) were inoculated beneath the myodesis with phosphate-buffered saline not containing bacteria (vehicle) and treatment rats were inoculated with $1 \times 10^6$ colony-forming units of *A baumannii* (n = 20) or MRSA (n = 20). All animals formed HO. Heterotopic ossification was determined by quantitative volumetric measurements of ectopic bone at 12-weeks postinjury using micro-CT and qualitative histomorphometry for assessment of new bone formation in the residual limb. Bone marrow and muscle tissue biopsies

This work was supported by USAMRAA BAA #10083004 (Principal Investigator JAF) and USAMRAA PRORP-CDMRP #120056 (Principal Investigator JAF).
All ICMJE Conflict of Interest Forms for authors and *Clinical Orthopaedics and Related Research*® editors and board members are on file with the publication and can be viewed on request.
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Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.
This work was performed at the Naval Medical Research Center, Silver Spring, MD, USA.

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were collected from the residual limb at 12 weeks to quantitatively measure the bioburden load and to qualitatively determine the species-level identification of the bacterial flora.

*Results* At 12 weeks, we observed a greater volume of HO in rats infected with MRSA ($68.9 \pm 8.6$ mm$^3$; 95% confidence interval [CI], 50.52–85.55) when compared with *A baumannii* ($20.9 \pm 3.7$ mm$^3$; 95% CI, 13.61–28.14; $p < 0.001$) or vehicle ($16.3 \pm 3.2$ mm$^3$; 95% CI, 10.06–22.47; $p < 0.001$). Soft tissue and marrow from the residual limb of rats inoculated with *A baumannii* tested negative for *A baumannii* infection but were positive for other strains of bacteria ($1.33 \times 10^2 \pm 0.89 \times 10^2$; 95% CI, $-0.42 \times 10^2$–$3.08 \times 10^2$ and $1.25 \times 10^6 \pm 0.69 \times 10^6$; 95% CI, $-0.13 \times 10^6$–$2.60 \times 10^6$ colony-forming units in bone marrow and muscle tissue, respectively), whereas tissue from MRSA-infected rats contained MRSA only ($4.84 \times 10^1 \pm 3.22 \times 10^1$; 95% CI, $-1.47 \times 10^1$–$11.1 \times 10^1$ and $2.80 \times 10^7 \pm 1.73 \times 10^7$; 95% CI, $-0.60 \times 10^7$–$6.20 \times 10^7$ in bone marrow and muscle tissue, respectively).

*Conclusions* Our findings demonstrate that persistent infection with MRSA results in a greater volume of ectopic bone formation, which may be the result of chronic soft tissue inflammation, and that early wound colonization may be a key risk factor.

*Clinical Relevance* Interventions that mitigate wound contamination and inflammation (such as early débridement, systemic and local antibiotics) may also have a beneficial effect with regard to the mitigation of HO formation and should be evaluated with that potential in mind in future preclinical studies.

## Introduction

Blast injuries present formidable surgical, treatment, and rehabilitation challenges. The resulting wounds are multifaceted, often resulting in composite tissue loss, comminuted open fractures, and frequent traumatic amputations. Related wound contamination is ubiquitous, often with multidrug-resistant organisms such as *Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* (MRSA), often calling for protracted treatment regimens that include serial surgical débridements and broad-spectrum antibiotic therapy [2, 3, 6]. A survey of wound infections from Combat Support Hospitals in Iraq from 2003 to 2004 demonstrated a relatively high frequency of MRSA (26%) followed by *Acinetobacter calcoaceticus-baumannii* complex (11%), *Klebsiella pneumoniae* (13%), and *Pseudomonas aeruginosa* (10%) in combat-related injuries [6]. Wound infection-related complications include wound dehiscence, deep soft tissue infection, biofilm development on orthopaedic implants, and infectious osteomyelitis, often leading to chronic, debilitating infections, further bone and soft tissue destruction, and subsequent limb amputation [2, 20, 22, 23, 33].

Heterotopic ossification (HO) is the formation of mature lamellar bone within soft tissue after severe traumatic injury [10]. It is known to develop in the majority of combat-related amputations, and early bacterial colonization has been considered a potential early risk factor [12, 13]. However, the cellular and early signaling mechanism(s) for combat injury-induced HO formation remain unclear. Recent findings suggest that the heightened and prolonged expression of inflammatory and other reparative mediators may be contribute to HO formation [11, 14]. Moreover, the combat wound appears to provide a unique microenvironment conducive to osteogenesis that promotes the skewed differentiation of endogenous tissue-derived progenitor cells toward ectopic bone development within injured and healing soft tissue [10].

We previously developed a rat model of combat-related HO that incorporates the critical elements associated with combat injury, specifically a systemic blast injury, femur fracture with soft tissue crush, and transfemoral amputation through the zone of injury wherein all animals develop radiographic evidence of HO within 2 months postinjury [25]. Expanding on this model, in this study, we sought to evaluate if (1) the presence of bioburden (*A baumannii* and MRSA) increases the magnitude of ectopic bone formation in traumatized muscle after amputation; and (2) what persistent effects bacterial contamination has on late microbial flora within the amputation site.

## Materials and Methods

### Animals

Forty-eight young adult pathogen-free male Sprague-Dawley rats (*Rattus norvegicus*; 12–14 weeks, 400–500 g) were purchased from Taconic Farms (Germantown, NY, USA). All animals were housed in clean plastic cages and kept on a 12-hour light/dark cycle with unlimited access to food (standard rodent chow) and fresh water ad libitum. The study protocol (12-OUMD-20s) was reviewed and approved by the Walter Reed Army Institute of Research/Naval Medical Research Center Institutional Animal Care and Use Committee in compliance with all applicable Federal regulations governing the protection of animals in research.

### Bacteria Culture Conditions

The *A baumannii* (strain 5075) and MRSA (MRSA strain 107261) organisms used in this study are highly virulent, well-characterized clinical specimens isolated from combat wounds from patients treated at the Walter Reed National Military Medical Center. In brief, frozen (−80 °C) stock cultures were streaked out on a blood agar plate and left to grow overnight at 37 °C and 5% $CO_2$. A single bacterial colony was isolated and suspended in 3 mL of Lysogeny broth/Luria-Bertabi medium (Becton, Dickinson and Co, Sparks, MD, USA) and agitated overnight at 37 °C and 5% $CO_2$. Overnight cultures were diluted 1:50 in 50 mL of fresh prewarmed Luria-Bertabi broth in a 250-mL Erlenmeyer flask and grown to early/midlog phase ($OD_{600}$ = 0.2–0.5) where cells proliferate in a logarithmic fashion under optimal culture and nutrient conditions resulting in a controlled cell growth rate. Next, 2 mL of the concentrated culture sample was removed. Cells were washed twice using prechilled (4°C) phosphate-buffered saline (PBS), pelleted by centrifugation (5000 rpm for 3 minutes), then resuspended in 1 mL of sterile PBS. The bacterial density was estimated through direct count using a Petroff-Hauser Counting Chamber (Hauser Scientific, Horsham, PA, USA) and confirmed by serial dilution and plating on Luria-Bertabi agar and then diluted to the desired cell concentration, $1 \times 10^7$ colony-forming units (CFU)/mL in cold PBS.

### Rat Model of Trauma-induced HO and Bacterial Inoculation

A total of 48 rats were exposed to blast overpressure exposure, femur fracture, soft tissue crush injury, and limb amputation as previously described [25]. After quadriceps myoplasty, three muscle sites immediately surrounding the amputation site were inoculated with: (1) vehicle (100 µL of PBS; n = 8); (2) *A baumannii* (100 µL of $1 \times 10^7$ CFU; n = 20); or (3) MRSA (100 µL of $1 \times 10^7$ CFU; n = 20). Closure of the incision was performed using a 3-0 Vicryl in the deep subcutaneous tissue and a running 4-0 subcuticular Monocryl. Wounds were covered in Vetbond (3M Animal Care Products, St Paul, MN, USA). Postoperatively rats were monitored at least twice daily by animal care staff, research investigators, and veterinarians for animal activity, signs of pain, weight loss, wound dehiscence, or infectious tracts for the duration of the study. Wounds that exhibited signs of infection defined as drainage, progressive marginal erythema, or dehiscence were débrided. Rats were euthanized if they demonstrated signs of infection after a third débridement. We conducted a power analysis based on the effect of a projected 50% increase in ectopic bone volume within the soft tissue surrounding the amputated femur when the injury site was inoculated with either MRSA or *A baumannii*. Using conservative assumptions and data from our prior studies, the power analysis showed that with eight rats per treatment group and $\alpha = 0.05$, there is 90% power to detect a 50% increase in ectopic bone volume. Thus, it was anticipated that 20 rats in the infected treatment groups would provide adequate statistical power to detect treatment effects of moderate size on the major outcome variable of ectopic bone volume even with attrition of as many as 12 rats per group. All eight rats in the control group survived until the 12-week micro-CT scan (Fig. 1). Six animals in the MRSA group were euthanized during the fourth and fifth weeks for overwhelming infection. Two of the rats in the *A baumannii* group died on the day of surgery and were excluded. A low level of mortality after surgery was consistent with findings during model development and represents the devastation of these multifaceted injuries, particularly given that blast overpressure of 120 ± 7 kPa itself is calibrated for 70% to 90% survivability [1, 7, 25]. In addition, two rats infected with *A baumannii* were euthanized for sustained weight loss greater than 10% during postoperative weeks 2 and 4.

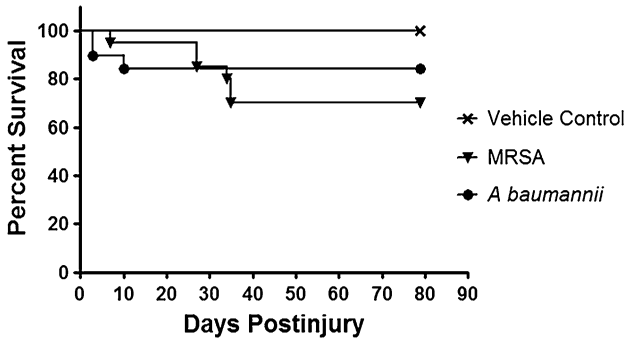


**Fig. 1** Treatment effects on survival outcome of injured rats wherein the traumatized muscle surrounding the amputation site at the time of closure was infected with either MRSA ($1 \times 10^6$) or *A baumannii* ($1 \times 10^6$). Kaplan-Meier survival curves are shown. Animals were euthanized if they demonstrated signs of infection after the third débridement and irrigation of the amputation wound site.

### Micro-CT Analysis

Rats anesthetized with isoflurane (2%) were imaged at 12 weeks postinjury using a SkyScan 1176 in vivo high-resolution micro-CT (Bruker-MicroCT, Kontich, Belgium) with the following settings: 89-kV polychromatic xray beam, current of 256 µA, and an exposure time of 81 msec for each of 180 rotational steps. Two investigators (GJP, ATQ) independently reviewed the micro-CT images (170–200 flattened longitudinal micro-CT slices/rat) on a CT-

Analyser (Bruker-MicroCT) and calculated the volume of ectopic bone formation using selected regions of interest on every fifth slice encompassing ectopic bone. The binary selected slices were then used to perform three-dimensional image analysis yielding a total volume of HO in the selected area of interest.

## Sample Collection and Culture

After the micro-CT scans were assessed for image quality and clarity, scanning efficiency, and reconstructed for volumetric analysis of ectopic bone formation, rats were euthanized with pentobarbital (Fatal Plus; 390 mg/kg intraperitoneally; Patterson Veterinary, Devens, MA, USA). Muscle tissue adjacent to the amputation site and femur was aseptically excised. Femurs were removed and separated from the soft tissues. Bone marrow from the residual femur was extruded from the medullary canal by flushing using a 10-mL syringe fitted with an 18-gauge needle with 10 mL of sterile PBS after proximal and distal osteotomies. Samples were diluted in PBS out to $10^{-6}$, plated on a blood agar plate, and incubated overnight at 37° C, 5% $CO_2$. Colonies were counted and screened for differing morphology. Isolates were streaked on a blood agar plate for direct bacterial species identification using the BD Phoenix automated microbiology system in accordance with the manufacturer's instructions (BD Diagnostics, Sparks, MD, USA).

## Histological Analysis

At the time of euthanasia, two rats from each treatment group received an en bloc resection of the residual limb, which was then fixed in 10% formalin, decalcified in 5% formic acid, paraffin-embedded, cut into 5-μm longitudinal sections on a microtome, and stained using hematoxylin and eosin stain (Histoserv, Inc, Germantown, MD, USA). Histologic tissue samples were qualitatively analyzed for evidence of soft tissue cartilage formation, inflammation, lamellar bone formation within the soft tissues, the presence of persistent inflammatory cells, or active bacterial infection. The histopathological analysis was conducted by a veterinary pathologist (CH) blinded to the treatment groups.

## Statistical Analysis

Kaplan-Meier modeling was performed to assess the survivability patterns of the control and treatment groups over the duration of the study. Intraclass correlation coefficient (ICC) was calculated to assess the reliability of interobserver measurements of HO formation using the micro-CT analyzing software. Analysis of variance modeling was used to determine whether there was a significant difference in the volume ($mm^3$) of ectopic bone measured among the three groups followed by the Tukey's honestly significant difference test to determine the mean difference among the three groups. All data, including the bacterial CFU counts, were presented as mean ± SD with 95% confidence interval (CI) unless specified otherwise. Exact p values were stated except when $< 0.001$. All statistical analysis described previously was performed using the RStudio, Version 0.98.953 (© 2009–2013 RStudio Inc, Boston, MA, USA).

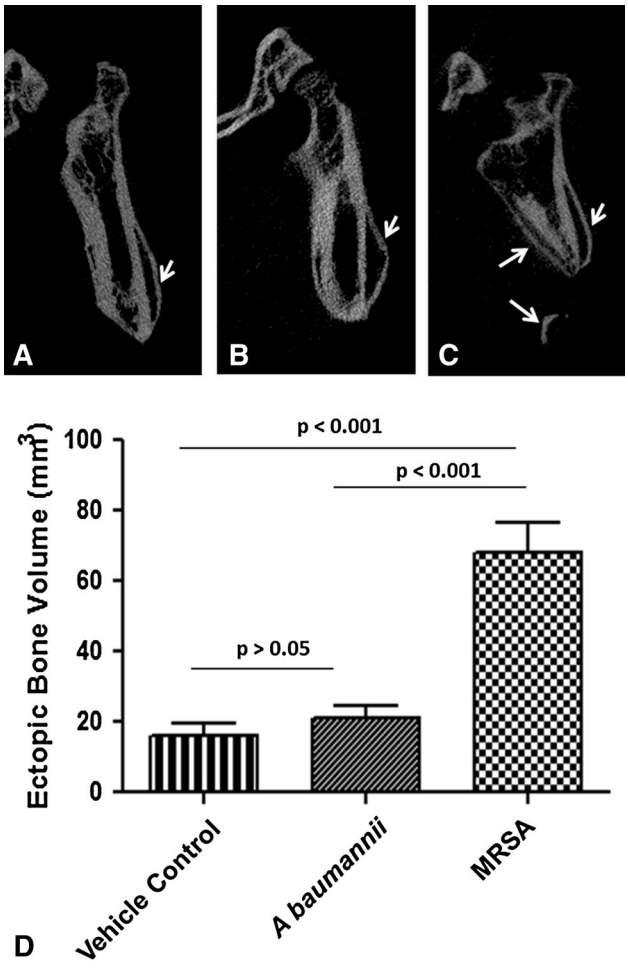


**Fig. 2A–D** MRSA infection increases trauma-induced ectopic bone formation. Representative longitudinal 12-week micro-CT images of the residual femurs of rats inoculated with (**A**) vehicle control (PBS; noninfected control); (**B**) *A baumannii*; and (**C**) MRSA are shown. The white arrows highlight the areas of ectopic bone formation. (**D**) The amount of ectopic bone was quantified 12 weeks postinjury from vehicle control (n = 8), *A baumannii* (n = 16), and MRSA (n = 14) treatment groups. Result expressed are expressed as the mean ± SD.

## Results

All rats that survived to the end of the study (*A baumannii* [n = 16], MRSA [n = 14], vehicle [n = 8]) demonstrated the formation of ectopic bone on the 12-week micro-CT scan (Fig. 2A–C). Volumetric measurements of ectopic bone formation (Fig. 2D) were more robust in animals inoculated with MRSA (68.0 ± 8.6 $mm^3$; 95% CI, 50.52–85.55) than *A baumannii* (20.9 ± 3.7 $mm^3$; 95% CI, 13.61–28.14; $p < 0.001$) and vehicle control (16.3 ± 3.2 $mm^3$; 95% CI, 10.06–22.47; $p < 0.001$). Comparison of vehicle control and *A baumannii*-inoculated groups showed no difference ($p = 0.43$) with excellent interobserver agreement (ICC = 0.98).

Soft tissue and bone marrow collected from the residual femur from rats inoculated with *A baumannii* tested negative for *A baumannii* flora but were positive for other strains of bacteria ($1.33 \times 10^2 \pm 0.89 \times 10^2$; 95% CI, $-0.42 \times 10^2$–$3.08 \times 10^2$ and $1.24 \times 10^6 \pm 0.69 \times 10^6$; 95% CI, $-0.13 \times 10^6$–$2.60 \times 10^6$ CFU in bone marrow and muscle tissue, respectively), whereas tissue from MRSA-infected rats contained MRSA only ($4.84 \times 10^1 \pm 3.22 \times 10^1$; 95% CI, $-1.47 \times 10^1$–$11.1 \times 10^2$ and $2.80 \times 10^7 \pm 1.73 \times 10^7$; 95% CI, $-0.60 \times 10^7$–$6.20 \times 10^7$) (Table 1). Specifically, bacterial culture results from the surviving MRSA-infected rats showed that in eight of 14 rats the muscle tissue surrounding the amputation site was positive for persistent MRSA infection, whereas five of the 12 bone marrow samples that were available after en bloc resection of two animals were MRSA-positive (Fig. 3). En bloc resection performed on MRSA rats demonstrated evidence of bacterial microcolonies, increased neutrophil infiltration, chronic soft tissue infection, and osteomyelitis (foci of bacterial microcolonies, purulent intramedullary infection, and evidence of bone necrosis indicative of empty osteocytic lacunae with islands of necrotic endochondral bone throughout the skeletal muscle; Fig. 4). All rats inoculated with *A baumannii* tested negative for the inoculated bacteria in both the soft tissue and bone marrow cultures. However, nine of 16 soft tissue samples and three of the 14 available bone marrow samples had positive cultures at 12 weeks with various bacterial flora species (Fig. 3). En bloc resection of *A baumannii* residual limbs sent for histology showed inflammatory cells indicative of chronic infection (data not shown); however, representative tissue sections (Fig. 4) failed to capture the periosteal reaction and ectopic bone formation observed on micro-CT (Fig. 2). No bacterial CFUs were detectable in the tissue cultures from vehicle-treated rats.

## Discussion

Blast injuries are devastating to the extremities and often include comminuted open fractures, neurovascular injury, soft tissue loss, and traumatic amputations. Wounds often are contaminated with foreign material and microorganisms, including *A baumannii* and MRSA, which have been proven to exhibit multidrug resistance or relatively high virulence, respectively [2, 6, 16, 21]. Because HO develops within the residual limbs of most blast- and otherwise combat-related amputations [3, 12, 13, 26], considerable efforts are directed toward treating symptoms conservatively; however, surgical resection is ultimately necessary

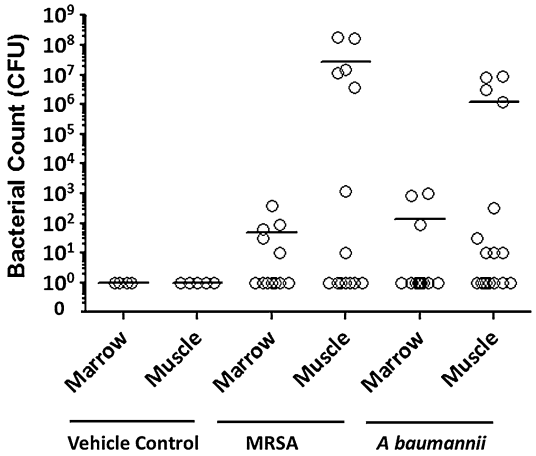


**Fig. 3** Bacterial titers (in CFUs converted to log scale) in the marrow compartment and soft tissue of rats infected with vehicle control (PBS; noninfected control), MRSA, and *A baumanni* after 12 weeks are shown. Each data point represents the actual CFU value for each animal in each treatment group, whereas the horizontal bar indicates the mean CFU for each treatment group. All rats inoculated with MRSA tested positive for MRSA, whereas rats inoculated with *A baumannii* tested positive for other microorganisms as detailed in Table 1.

**Table 1.** List of bacteria present in the marrow compartment and soft tissue 12 weeks postinjury

| Tissue | Vehicle control | *Acinetobacter baumannii* | Methicillin-resistant *Staphylococcus aureus* |
|---|---|---|---|
| Marrow | Negative | *Arcanobacterium haemolyticum*; *Enterobacter cloacae* and *Enterococcus faecalis* | *S aureus* |
| Muscle | Negative | *Arcanobacterium haemolyticum, Streptococcus porcinus, Staphylococcus cohnii ssp urealyticum, Staphylococcus xylosus, Gardnerella vaginalis, Pasteurella multocida, Enterobacter cloacae,* and *Enterococcus faecalis* | *S aureus* |

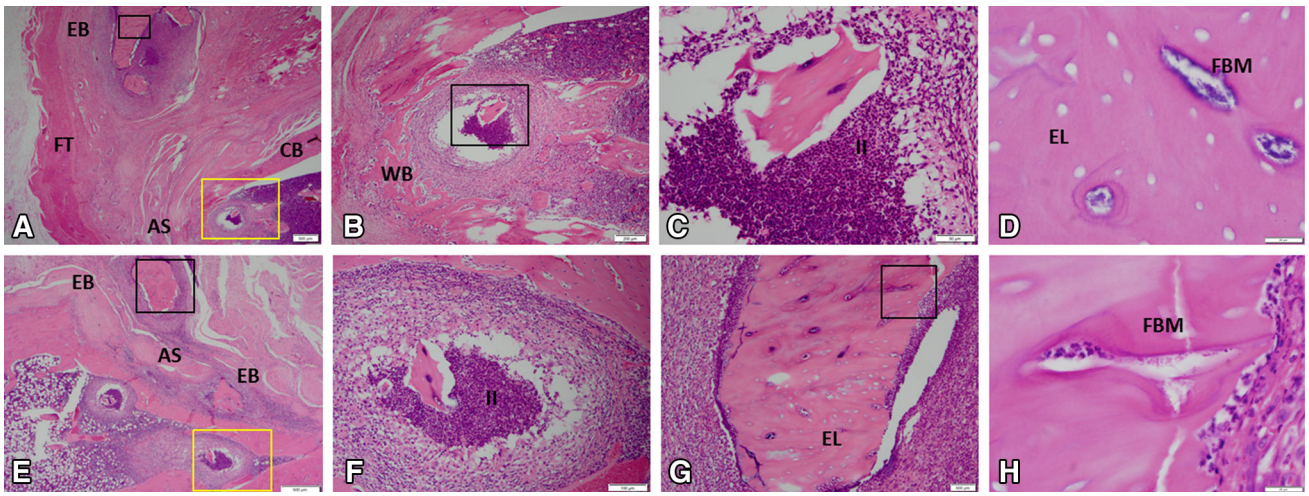


**Fig. 4A–H** The histological features of ectopic bone formation in MRSA-treated rats at 12 weeks are shown in **A**–**H** (**A**, Stain, hematoxylin and eosin; **A** original magnification, × 1.25; **B**, original magnification, × 4, yellow boxed region in **A**; **C**, original magnification, × 20, black boxed region in **B**; **D**, original magnification, × 100, black boxed region in **A**; **E**, original magnification, × 2; **F**, original magnification, × 10, yellow boxed region in **E**; **G**, original magnification, × 10, black boxed region in **E**; **H**, original magnification, × 10, black boxed region in **G**). For detailed evaluation, images of six selected regions at higher magnification are shown. In the medullary space and soft tissue, there is evidence of chronic inflammation, neutrophil infiltration, purulent infection, osteomyelitis, and necrotic ectopic bone as indicative of empty osteocytic lacunae containing bacterial microcolonies. AS = amputation site; CB = cortical bone; EL = empty lacunae; EB = ectopic bone; FBM = foci of bacterial microcolonies; FT = fibroblastic tissue; II = intramedullary infection; WB = woven bone.

in up to 41% of amputees with HO [30]. As such, considerable focus has been directed toward prevention and mitigation of HO formation; however, understanding factors that exacerbate its development is an important prerequisite. In this effort, we explored, using an established blast-related HO animal model [25], the impact of *A baumannii* or MRSA colonization on the volume of HO formation and identified the characteristics of chronic infection in each setting.

There are several limitations to our study. First, the rat model is not conducive to many of the surgical modalities used in the treatment of traumatic wounds such as serial débridements with negative pressure wound therapy, which are implicated as putative contributors to HO formation [12]. Second, most war wounds are typically colonized by polymicrobial flora [6]. As such, an inoculum of a specific bacterial pathogen ($1 \times 10^6$ CFU) does not fully address the synergistic role that polymicrobial infection may have in the persistence and virulence of infection plus it limits our ability to assess differences in HO formation or persistence of infection with varied degrees of infection. Preliminary experiments demonstrated that the bacterial concentration of MRSA used in these studies resulted in established persistent infections with high reproducibility and minimal variation in regard to wound complications. Moreover, it has been reported that approximately 50% of combat wounds become clinically infected ($> 1 \times 10^5$ CFU) as opposed to merely contaminated [2, 28]. Notably, as a limitation in identifying the presence of all persistent microorganisms, only aerobic wound microflora were cultured from soft tissue and bone marrow. With the expressed intent of describing the impact of microbial bioburden on trauma-induced HO, we acknowledge our limited description of other forms of HO such as genetic and neurogenic. Neurogenic HO has been well described in civilian populations [8, 15], whereas the focus in military research has been predominantly in traumatic HO. In the neurogenic form, neurotransmitters such as glutamate, substance P, and catecholamines act to induce osteoblasts to form ectopic bone within a permissive local environment [5, 18, 27]. Therefore, the induction of progenitor cells with varying osteoinductive factors is common in both traumatic and neurogenic; however, the difference lies in the elevated levels of systemic and local inflammatory cytokines in the former and neurotransmitters in the latter [14]. That having been said, the expression and/or production of inflammatory mediators in this current study was not assessed; therefore, inferences regarding the role of local infection on HO development are based only on histopathological changes noted at study termination.

Contamination of residual limbs with MRSA, but not *A baumannii,* contributed to the volume of HO that developed in this rat model. This finding is relevant given that MRSA is the predominant organism in 35% to 50% of clinically infected combat wounds [3, 4]. Often, these wounds demand serial débridements to achieve healthy-appearing and/

or culture-negative tissue. Despite this, approximately one-fourth of amputations develop late infection after closure of a healthy-appearing wound bed [30]. More débridements, five to seven to be exact, are associated with the development of HO, likely resulting from mechanical trauma to the tissue as well as the systemic inflammatory responses that can result from repeated returns to the operating room [7, 19]. In comparison, our MRSA-inoculated rats developed a greater volume of HO with most (11 of 14 analyzed) doing so in the absence of the described serial surgical interventions, further suggesting MRSA involvement. An unexpected finding of our study is the relative lack of effect of *A baumannii* on ectopic bone formation despite the selection of the strain based on its clinical ubiquity and relative virulence [2, 29]. This result may be expected given that *A baumannii*-infected rats cleared their infection. Another explanation involves the signaling of toll-like receptors (TLRs), which are found to be expressed on osteogenic precursor cells [24]. Interestingly, purified lipopolysaccharide, a ligand found on Gram-negative organisms, which has preferential affinity for toll-like receptor 4 (TLR4), demonstrated slow activation of mesenchymal stem cells; however, prolonged exposure to the toxin resulted in decreased expression of TLRs. Alternatively, downregulation of TLRs did not occur with prolonged exposure to the Gram-positive specific cell wall component lipoteichoic acid, perhaps allowing for osteogenic differentiation of mesenchymal stem cells by Gram-positive organisms like MRSA [19, 31]. It may be that infection with Gram-negatives such as *A baumannii* affects HO development indirectly in clinical practice. This organism is not found in wounds at the time of injury but rather is a nosocomial pathogen found in combat theater hospitals. Infection of wounds during aeromedical evacuation or at combat hospitals may "reinfect" wounds, resulting in serial débridements and negative pressure wound therapy, factors that some infer may contribute to HO development [6, 12, 23].

Chronic infection, like persistently symptomatic HO, can delay or regress the rehabilitation of blast- and otherwise war-related amputees. After combat-related lower extremity amputations, 27% require return to the operating room for wound infection [30] at some point during their hospitalization. In a study of 110 service members with severe orthopaedic wounds that subsequently developed osteomyelitis, a retrospective review showed that *A baumannii* accounted for 70% of initial admissions to the hospital; however, these responded well to treatment, making up only 6% of recurrences. By comparison, MRSA, presented as the infecting organism in only 8% of initial diagnoses of osteomyelitis, however, was responsible for 31% of readmissions for osteomyelitis with Gram-positives as a whole accounting for 75% of recurrence [33]. In addition, modeling of war wounds in a rabbit demonstrated that monobacterial Gram-negative inoculation at a titer of $1 \times 10^5$ *A baumannii* failed to produce osteomyelitis, whereas polymicrobial inoculum and/or those containing MRSA demonstrated active, persistent, infection 8 weeks postinoculation [32]. Our findings also support the persistence of Gram-positive infections with 56% and 21% rates of soft tissue contamination and osteomyelitis in our MRSA cohort, the latter occurring despite the bone not being directly inoculated. Conversely, *A baumannii* was not present in any 12-week cultures, but rather other antibiotic-resistant ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus, K pneumoniae, A baumanii, P aeruginosa*, and *Enterobacter* species) such as enteric organisms *Enterococcus faecium* and *Enterobacter cloacae* as well as various Gram-positive staphylococci, but not *S aureus* were isolated at time of culture (Table 1). Negative culture results for *A baumannii* infection 12 weeks postinoculation are consistent with previous rat studies and may be the result of decreased virulence in bone as compared with other infection sites [9]. Secondary infection with other nosocomial pathogens, particularly Gram-positive organisms, is consistent with clinical findings and suggests that initial infection with *A baumannii* may produce an environment conducive to secondary infection or overgrowth of other nosocomial organisms [17]. Although the synergism between initial *A baumannii* infection and subsequent infection still needs to be studied, this result may be informative for clinical treatment plans.

Our study suggests that of the two most common bacterial isolates of combat-related amputations, MRSA infection results in the development of a several-fold increase in the volume of ectopic bone compared with *A baumannii* and a vehicle control in a rat model. This difference may be related to the microorganisms' persistent colonization and invocation of chronic infection because this difference was shown in our study minus the surgical treatment already known to influence HO formation. Therefore, in addition to drug therapies that target signaling pathways in bone development and/or proinflammatory osteogenic mediators, we further propose that initiation of prophylactic local and/or systemic Gram-positive antimicrobial therapy at the time of injury and continued treatment of subclinical infection may help mitigate the formation of ectopic bone; further preclinical work, to include assessment of polymicrobial infections, impact of differential TLR signaling, and the evaluation of systemic and/or local antimicrobial interventions, is necessary to further elucidate this effect.

**Acknowledgments** We thank Dana Golden, Allison Tomasino, and Erica Crump for assistance with animal care, sample collections, and technical expertise. We also thank Ying Cao for his statistical expertise and assistance. Furthermore, we thank LTC Cary Hannold for his excellent contribution to the histological review of tissue specimens.

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Clin Orthop Relat Res (2015) 473:2831–2839
DOI 10.1007/s11999-015-4240-y

Clinical Orthopaedics and Related Research®
A Publication of The Association of Bone and Joint Surgeons®



SYMPOSIUM: RESEARCH ADVANCES AFTER A DECADE OF WAR

# Early Characterization of Blast-related Heterotopic Ossification in a Rat Model

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Published online: 10 March 2015


**Abstract**

*Background* Heterotopic ossification (HO) affects the majority of combat-related lower extremity wounds involving severe fracture and amputation. Defining the timing of early osteogenic-related genes may help identify candidate prophylactic agents and guide the timing of prophylactic therapy after blast and other combat-related extremity injuries.

*Questions/purposes* Using a recently developed animal model of combat-related HO, we sought to determine (1) the timing of early chondrogenesis, cartilage formation, and radiographic ectopic bone development; and (2) the early cartilage and bone-related gene and protein patterns in traumatized soft tissue.

*Methods* We used an established rat HO model consisting of blast exposure, controlled femur fracture, crush injury, and transfemoral amputation through the zone of injury. Postoperatively, rats were euthanized on Days 3 to 28. We assessed evidence of early ectopic bone formation by micro-CT and histology and performed proteomic and gene expression analysis.

*Results* All rats showed radiographic evidence of HO within 28 days. Key chondrogenic (*collagen type I alpha 1* [*COL1α1*], p = 0.016) and osteogenic-related genes (*Runt-*

This work was supported by CDMRP (W81XWH-14-2-0010; PI-JAF) and BUMED (602115HP.3720.001.A1014; PI-JAF).


**Electronic supplementary material** The online version of this article (doi:10.1007/s11999-015-4240-y) contains supplementary material, which is available to authorized users.

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*related transcription factor 2* [*RUNX-2*], p = 0.029; *osteoclacin* [*OCN*], p = 0.032; *phosphate-regulating neutral endopeptidase, X-linked* [*PHEX*], p = 0.0290, *and POU domain class 5 transcription factor* [*POU5F*], p = 0.016) and proteins (*Noggin* [*NOG*], p = 0.04, *OCN*, p = 0.02, *RUNX- 2*, p = 0.04, and *substance P-1* [*SP-1*], p = 0.01) in the injured soft tissue, normalized to the contralateral limb and/or sham-treated naïve rats, increased on Days 3 to 14 postinjury. By 14 days, foci of hypertrophic chondrocytes, hyaline cartilage, and woven bone were present in the soft tissue surrounding the amputation site.

*Conclusions* We found that genes that regulate early chondrogenic and osteogenic signaling and bone development (*COL1α1, RUNX-2, OCN, PHEX,* and *POU5F1*) are induced early during the tissue reparative/healing phase in a rat model simulating a combat-related extremity injury.

*Clinical Relevance* The ability to correlate molecular events with histologic and morphologic changes will assist researchers and clinicians to understand HO and hence formulate therapeutic interventions.

## Introduction

Heterotopic ossification (HO) refers to the abnormal development of bone in nonosseous tissue, most commonly occurring in the setting of orthopaedic trauma, severe burns, neurotrauma, or major surgery [2, 7, 10, 13]. The prevalence of HO in the residual limbs of returning service members with combat-related amputations is reported to be as high 65% [11, 22]. At least 41% of those patients who develop HO require additional excision procedures. Moreover, delayed healing and wound dehiscence are major problems in severely injured patients recovering from survivable severe battlefield blast-related extremity injuries [12]. Studies from our group have established that acute wound failures and subsequent HO formation are related to multiple complex interrelated systemic and local inflammatory responses to traumatic injury [8, 12]. The optimal treatment strategy for HO has not been defined. Surgical excision is the only definitive management option and treatment of symptomatic HO if physical therapy and prosthesis alteration fail to provide adequate relief [31]. Other prophylaxis strategies include treatment with nonsteroidal antiinflammatory drugs or external-beam radiotherapy, but these options are more confined in the civilian setting and are generally contraindicated in the setting of combat and blast-induced trauma given nonsteroidal anti-inflammatory drugs may delay fracture healing and cause unacceptably high rates of bleeding complications while radiotherapy must be administered within 48 hours of injury (difficult if not impossible in the combat setting) and is known to cause wound- and implant-related complications [11, 22, 23].

A comprehensive assessment of the early histologic and molecular development processes present within blast wounds has not occurred for several reasons. First, clinically relevant animal models have only recently been developed [21, 30]. Second, clinical diagnostic methods are unable to accurately predict the site of HO development [5, 20]. Third, analysis of human tissues obtained early in the débridement process has not been performed. Finally, available human clinical samples, usually collected at the time HO excision, typically contain immature and mature bone. Defining the early development phase of HO in relationship to concurrent wound healing is critical to selection of candidate means of prophylaxis and, importantly, the timing of their administration after high-energy extremity injuries.

We developed a rat model of combat-related HO that incorporates the critical elements associated with combat injury, specifically a blast injury, femur fracture-crush, and transfemoral amputation, through the zone of injury wherein all animals develop radiographic evidence of HO within 2 months postinjury [21]. In this report, we use our model to address (1) the timing of early chondrogenesis, cartilage formation, and radiographic ectopic bone development; and (2) the early cartilage and bone-related gene and protein patterns in traumatized soft tissue subsequent to calcium deposition, tissue mineralization, and ectopic bone formation. It is important to confirm the timing and upregulation of bone-related genes and proteins in our model because some observational clinical studies with soft tissue injury (without fracture or amputation) have shown elevated levels of such genes in a minority of cases [6, 9, 12].

## Materials and Methods

### Animals

Fifty-four young adult pathogen-free male Sprague-Dawley rats (*Rattus norvegicus*; 400–500 g) were purchased from Taconic Farms (Germantown, NY, USA). All animals were housed in clean plastic cages and kept on a 12-hour light/-dark cycle with unlimited access to food (standard chow) and fresh water ad libitum. The study protocol (12-OUMD-20s) was reviewed and approved by the Walter Reed Army Institute of Research/Naval Medical Research Center Institutional Animal Care and Use Committee in compliance with all applicable Federal regulations governing the protection of animals in research. Postoperatively, rats were monitored at a minimum twice daily for animal activity, signs of pain, wound dehiscence, weight loss, and infection

by animal care staff, research staff, and veterinarians. Moribund rats were euthanized.

### Rat Heterotopic Ossification Model

Rats were anesthetized with isoflurane (2%–5%) and received full-body blast overpressure (120 ± 7 kPa) exposure, without any shielding to the blast wave, through a pneumatically driven shock tube [1, 4, 29]. Preoperative buprenorphine (0.05 mg/kg) was then administered through intraperitoneal injection and a reproducible comminuted fracture of the right femur was performed using a drop weight apparatus from a height of 88 cm. A crush injury was performed immediately after the fracture by rotating the fracture site between the two support anvils, generating 20 pounds per square inch of pressure for 1 minute [3]. The injured limb was then amputated through the fracture with appropriate hemostasis and débridement of devitalized tissue followed by hamstring and quadriceps myoplasty over the exposed residual femur. Postoperative pain was managed with sustained-release buprenorphine (1.2 mg/kg) administered subcutaneously with repeat dosing after 3 days. Cohorts of four to eight rats per time point were euthanized on postinjury Days 3, 5, 7, 10, 14, 21, and 28 to detect and visualize histologically the early stages of the HO disease process, whereas a cohort of four sham-treated (neither blasted nor injured) naïve rats euthanized on Day 3 served as controls. Two rats were euthanized postoperatively early for self-mutilation of the amputation site and one for consumption and subsequent suffocation as a result of inhalation of the bedding. A surgical team consisting of an orthopaedic surgeon (GJP, EKC or DNH), postdoctorate fellow (ATQ), and two surgical technicians (AMT, DMG) experienced in small animal anesthesia and surgeries conducted the experiments and immediate postoperative care procedures.

### Micro-CT for Detection of Ectopic Bone Formation

Postoperatively rats were anesthetized with isoflurane (2%–5%) and the injured leg was imaged using a SkyScan in vivo 1176 high-resolution micro-CT (lCT) x-ray imaging in three dimensions (Bruker-MicroCT, Kontich, Belgium) with the following settings: 89-kV polychromatic x-ray beam, current of 256 μA, and an exposure time of 81 milliseconds for each of 180 rotational steps. The three-dimensional (3-D) images were rendered to reconstruct tomograms with a commercial package (NRecon; Bruker-MicroCT).

### Tissue Collection for Chondrogenic, Osteogenic and Angiogenic Gene Expression and Protein Analysis

After euthanasia, skeletal muscle was aseptically collected from the distal quadriceps of the amputated limb as well as from the distal quadriceps muscle of the contralateral limb. Samples were immediately placed in either RNAlater™ (Ambion Inc, Austin, TX, USA) at 4 °C for 48 hours or snap-frozen in dry ice for gene expression and protein analysis, respectively.

### Histological Analysis

The residual injured and contralateral femurs with attached associated muscle tissue were surgically removed and placed in 10% formalin. The femurs were decalcified in 5% formic acid, embedded in paraffin, longitudinal sectioned (5 μm), and stained with hematoxylin-eosin (Histoserv, Inc, Germantown, MD, USA). Qualitative observations of wound healing and early ectopic endochondroal bone development (mesenchymal condensation, chondrocyte differentiation, chondrogenesis, hypertrophic vascularized cartilage, hyaline cartilage development, extracellular maturation, and soft tissue mineralization) were performed by a pathologist (CLH) who was blinded to the study.

### RNA Isolation and Gene Expression

Total RNA was isolated from skeletal muscle samples as previously described [9]. A custom-made low-density reverse transcription-polymerase chain reaction (RT-PCR) array consisting of 96 primer sets (including respective forward and reverse primers) for 83 rat-specific osteogenic, chondrogenic, adipogenic, and angiogenic genes as well as six housekeeping and seven quality control genes (SABiosciences, Gaithersburg, MD, USA) was used to assess gene expression (genes and their function listed in Supplemental Table 1 [Supplemental materials are available with the online version of CORR®.]). Quantitative RT-PCR and dissociation curve analyses were performed as previously described [9]. Cycle threshold (Ct) measurements per samples were normalized using *GAPDH*. Relative expression between sham-treated naïve rats and the injured limb muscle samples was determined using the comparative Ct method ($2^{-\Delta\Delta Ct}$) [17]. In comparison to sham-treated naïve rats, genes that were deferentially expressed at least threefold were depicted using a heat map. Assays with Ct values greater than 35 cycles were considered not expressed and are not reported.

### Quantification of Protein Expression

A sample (detailed subsequently) of the differentially expressed genes involved in extracellular matrix remodeling, cartilage deposition and vasculogenesis, and mineralization-ossification were verified by enzyme-linked immunosorbent assay (ELISA) [24]. Protein from skeletal muscle samples (30–32 mg) of the injured and contralateral limbs of rats euthanized 3, 5, and 7 days postinjury was isolated using the Total Protein Extraction Kit (EMD Millipore, Billerica, MA, USA) and total concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Quantification of protein levels of rat substance P (*SP-1*), Neurokinin A, and calcitonin gene-related peptide (*CGRP*) were assayed by enzyme immunoassay (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA). In addition, levels of noggin (*NOG*), osteocalcin (*OCN*), runt-related transcription factor 2 (*RUNX-2*), and bone morphogenetic protein 2 (*BMP-2*) were assayed by ELISA (MyBioSource, San Diego, CA, USA). For each analyte, samples were equally loaded based on the total protein concentration assayed in duplicate. Absolute tissue sample concentrations of each analyte were calculated from a standard curve of known standards and corrected for protein concentration.

### Statistical Analysis

Continuous variables (protein expression) were evaluated with Student's t-test provided the data were normally distributed, whereas noncontinuous data (gene expression) were analyzed with the Mann-Whitney U test. Two-tailed $\alpha < 0.05$ was considered statistically significant. All data are presented as means ± SD unless otherwise specified.

## Results

In this physiologic model of combat-related HO, blast exposure in the presence of severe extremity trauma produced μCT radiographic evidence of HO within the soft tissue surrounding the fracture/amputation site in 100% of the animals within 28 days (Fig. 1). We observed no radiographic evidence of neurogenic HO development (around joints and/or in the soft tissue distant from the fracture/amputation site) in our model or in blast only-treated rats. Foci of proliferative/hypertrophic chondrocytes were observed in tissue surrounding the amputation site (Fig. 2A–F) as early as 5 days and certainly by postoperative Day 10. By Day 14, endochondral ossification was evident because the ectopic chondrocyte-rich basophilic hyaline cartilage was replaced with acidophilic bone matrix (osteoid) later followed by the immature woven bone typical of HO arising from the process of endochondral ossification (Fig. 2G–I). None of the contralateral limbs from blast-injured rats or limbs from sham-treated rats developed radiographic or histologic evidence of HO.

Genes involved in chondrogenesis (*COL1α1*), osteogenesis (*RUNX-2, OCN, PHEX,* and *POU5F1*), wound healing/tissue repair (*MMP9, CSF3, FGF-10,* and *HAS1*), and adipogenesis (*ADIPOQ* and *PPARG*) were notably overexpressed (greater than threefold) at the amputation site, whereas all angiogenic targets were unchanged (less than threefold) in comparison to quadriceps muscle tissue collected from the contralateral limb and sham-treated naïve rats (Fig. 3). The in vivo tissue production of key osteogenic proteins *NOG* (6.78 ± 1.38 ng; 95% confidence interval [CI], 4.06–9.50; $p = 0.04$), *OCN* (6.54 ± 0.56 ng; 95% CI, 5.43–7.65; $p = 0.02$), and *RUNX-2* (7.76 ± 0.94 ng; 95% CI, 5.92–9.61; $p = 0.04$) were elevated at 3 days postinjury relative to normal muscle tissue collected from sham-treated 5.92–9.61 naïve controls (Fig. 4A). In addition, we observed that the amount of the peripherally released neurotransmitter

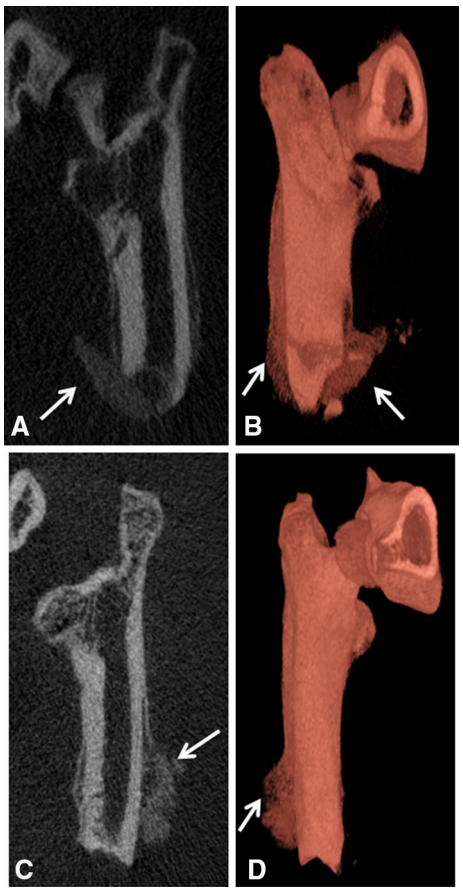



**Fig. 1A–D** Representative μCT and 3-D reconstructed images of rats euthanized at postinjury Day 21 (**A**–**B**) and Day 28 (**C**–**D**) are shown. The arrows indicate the formation of ectopic bone.

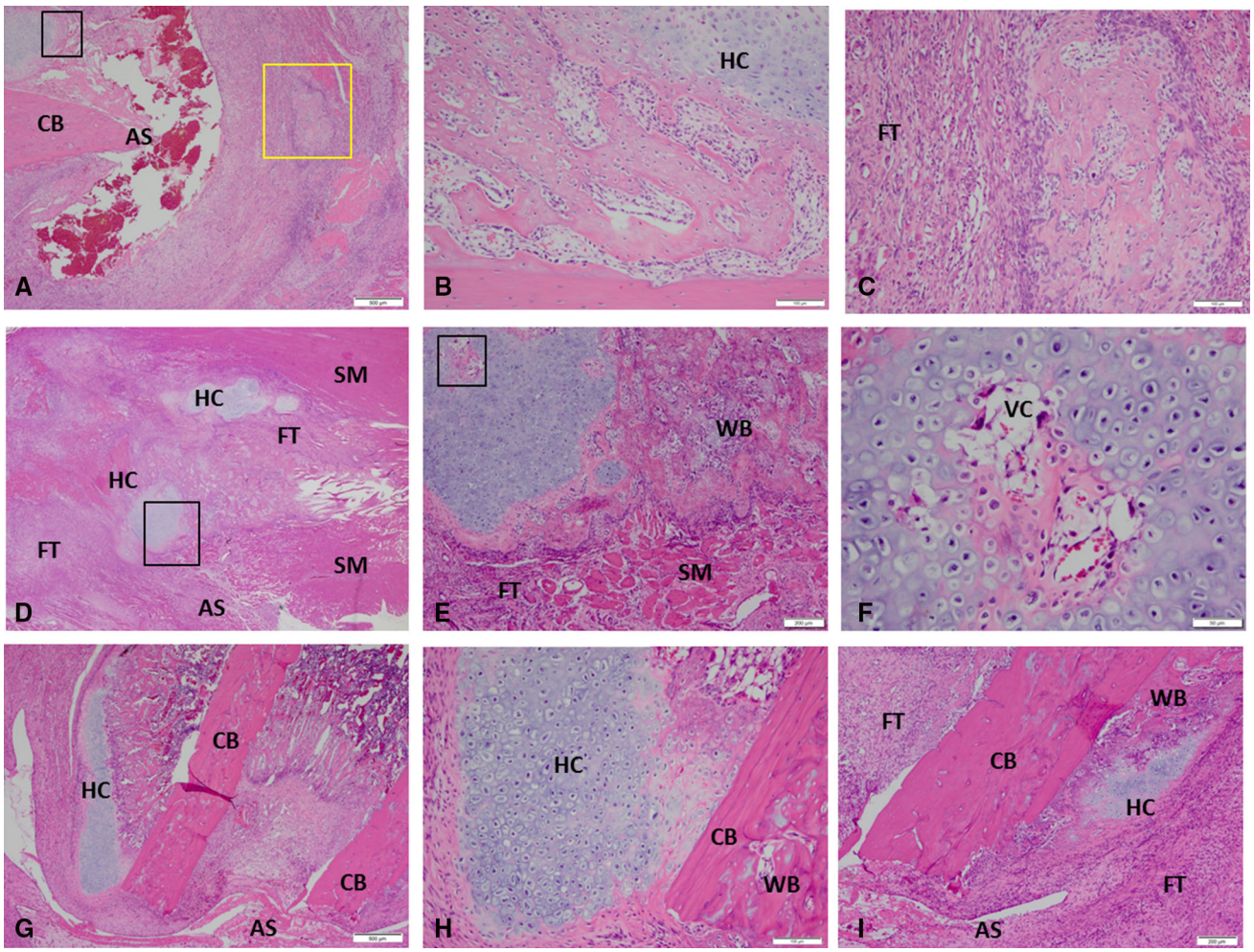



**Fig. 2A–I** These images show the histological evidence of early HO formation at the site of amputation postinjury Day 7 (**A**–**C**), Day 10 (**D**–**F**), and Day 14 (**G**–**I**) (Stain, hematoxylin and eosin). For detailed evaluation, higher magnification images of five selected regions are shown. (**B**–**C**) High-magnification views of the areas are outlined by the black inset box and yellow inset box in **A**, respectively. (**E**, **F**, **H**) High-magnification views show the areas outlined the black inset box in **D**, **E**, and **G**, respectively. Foci of hyaline and vascularized cartilage with woven bone are observed in the soft tissue surrounding the site of amputation at postinjury Days 10 to 14. AS = amputation site; CB = cortical bone; FT = fibroblastic tissue; HC = hyaline cartilage; SM = skeletal muscle; VC = vascularized cartilage; WB = woven bone.

*SP-1* was higher in the injured limb at 3 to 7 days postinjury (0.2 ± 0.04 ng; 95% CI, 0.10–0.27; 0.32 ± 0.10 ng; 95% CI, 0.10–0.53; and 0.4 ± 0.13 ng; 95% CI, 0.12–0.67; per 30 mg of tissue) when compared with sham-treated naïve control muscle ($p = 0.002$, $p = 0.009$, and $p = 0.01$, respectively) and muscle collected from the contralateral leg, which was subjected only to blast-related trauma (Fig. 4B). There were no differences observed in concentrations of neurokinin A, *CGRP*, or *BMP-2* (data not shown).

## Discussion

High-energy blast exposure to the extremities results in extensive soft tissue, muscle, vascular, nerve, and bone destruction often resulting in limb amputations, which collectively pose formidable surgical, postoperative treatment, and rehabilitation challenges. The prevalence of ectopic bone development in the residual limbs of patients with combat-related amputations is reported to be as high 65% [22]. Advances in combat casualty care-related wound healing are limited by an incomplete understanding of fundamental cellular and molecular mechanisms driving normal healing processes versus the dysregulated wound healing responses. In this study, we described the histology and local microenvironment during the early differentiation phase of stem cells/progenitor cells in a rat model of combat-related HO that integrates some of the key injury patterns seen in our blast-wounded service members. Using our established and highly reproducible animal model of trauma-induced HO, we provide definitive evidence that

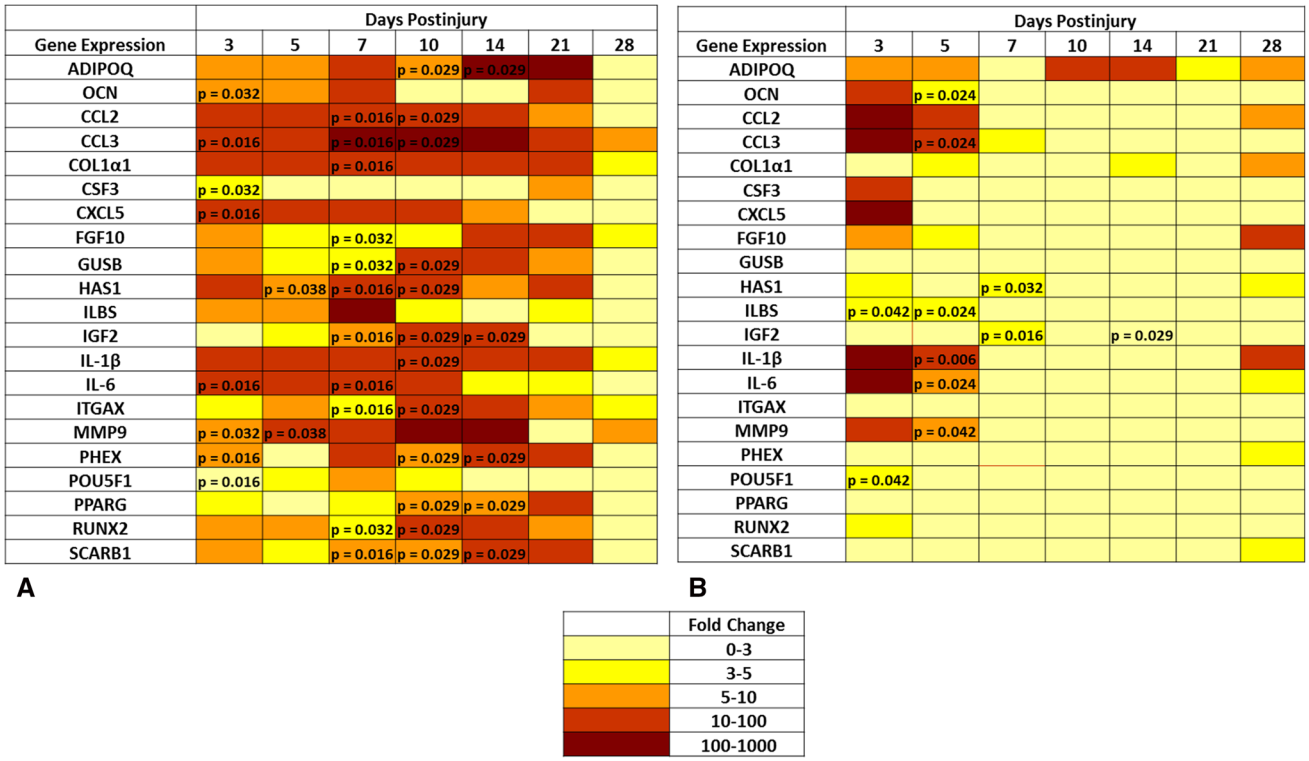
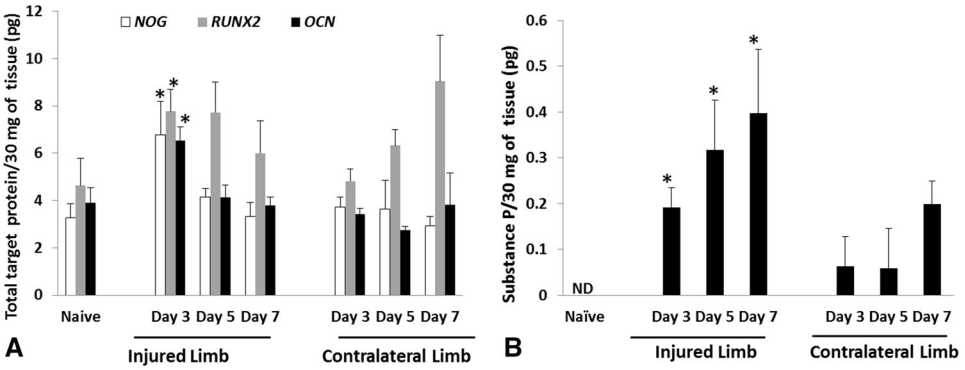



**Fig. 3A–B** A transcript heat map depicting the expression level of the subset of the 83 rat chondrogenic, osteogenic, and angiogenicrelated gene targets whose differential expression was greater than threefold compared with the expression level in shamtreated control muscle. (A) Injured leg and (B) contralateral leg and significantly different ($p < 0.05$; Mann-Whitney U test) compared with sham-treated rats are noted with p values.

the pathophysiological process of ectopic bone formation is through endochondral ossification, replacement of cartilage by bone. Results from these studies that recapitulate the clinical disease process will be most useful in advancing our understanding of early underlying molecular signaling pathways and cell development stages involved in formation of extraskeletal bone formation and facilitate the identification of targeted novel therapeutic strategies.

Several limitations to our study are noteworthy. The primary limitation is that the work was only conducted on one animal species. It is possible that a large animal model (swine or nonhuman primate), similar in size and physiology to humans, may more closely mimic the trauma-induced local and systemic responses exhibited by combat casualties. In addition, larger animal models may allow for the incorporation of other postoperative surgical and treatment variables such as serial débridement procedures and negative pressure wound therapy, which is difficult to use in a small animal model. Second, factors that influence the development of HO may prove to be the actual biological mechanism accounting for the extent and severity of injury. Unlike on the battlefield, the multifaceted injury patterns, limb amputation, and surgical repair/wound closure in this model were all made in sequence and within hours of injury rather than days to weeks postinjury after serial débridement procedures, as seen in the clinical setting. Third, we assessed the combinatorial effects of the critical elements associated with combat injury, which results in 100% radiographic evidence of ectopic bone formation. In regard to early histological changes and gene expression signaling, it may be worthwhile to evaluate the importance of each injury pattern alone and in various combinations. Lastly, we evaluated the time course of gene expression for a small subset of genes at given times wherein regulation may have occurred earlier than 3 days postinjury and/or expression of some genes may be highly temporal in regulation during early ectopic bone development. Furthermore, it is likely that the same mediators that promote normal wound healing also support ectopic bone development: however, we believe the nature and severity of the injury involving blast exposure in conjunction with a heightened and prolonged local and systemic immune response plays a major role in ectopic bone formation/wound dehiscence versus normal healing in combat-wounded patients.

Micro-CT scans showed an increase in ectopic bone development at 3 to 4 weeks within the soft tissue surrounding the site of amputation. These findings are consistent with those of radiographic studies detecting




C

| | Mean | SD | 95%CI | p Value |
|---|---|---|---|---|
| NOG - Naive | 3.25 | 1.04 | 2.07-4.43 | |
| NOG – Day 3 Injured Limb | 6.78 | 2.40 | 4.06-9.50 | 0.04 |
| | | | | |
| OCN - Naive | 3.89 | 1.30 | 2.61-5.17 | |
| OCN – Day 3 Injured Limb | 6.54 | 0.80 | 5.43-7.65 | 0.02 |
| | | | | |
| RUNX2 - Naive | 4.63 | 1.99 | 2.37-6.89 | |
| RUNX2 – Day 3 Injured Limb | 7.76 | 1.33 | 5.92-9.61 | 0.04 |
| | | | | |
| SP-1 - Naive | ND | ND | ND | |
| SP-1 – Day 3 Injured Limb | 0.19 | 0.08 | 0.10-0.27 | 0.002 |
| SP-1 – Day 5 Injured Limb | 0.31 | 0.21 | 0.10-0.53 | 0.009 |
| SP-1 – Day 7 Injured Limb | 0.39 | 0.27 | 0.12-0.67 | 0.01 |

**Fig. 4A–C** The amount of *NOG, RUNX-2* and *OCN* (**A**) and *SP-1* (**B**) protein was quantified from tissue samples harvest at the site of amputation. (**C**) The levels of *NOG*, *OCN,* and *RUNX-2* are statistically significant from the sham-treated (naïve) rats at 3 days postinjury, whereas the levels of *SP-1* are significantly different from the sham-treated (naïve) rats at 3 to 7 days postinjury (*$p < 0.05$; Student's t-test).

early evidence of soft tissue mineralization and bone deposition in combat-wounded patients [23]. At Day 7 to 10 postinjury, abundant foci of hypertrophic chondrocytes and vascularized hyaline cartilage were present. By Days 10 to 14, the soft tissue contained abundant hyaline cartilage with hypertrophic chondrocytes with evidence of subsequent mineralization and osteoprogenitor cell migration as denoted by zones of primary woven bone (immature endochondral bone formation. By Day 28, qualitative analysis of ectopic bone formation using 3-D high-resolution μCT showed a peak in the size of radiographically detectable bone in the soft tissue, which was supported by clear histological evidence of woven bone. This is similar to the results observed in another animal model of post-traumatic HO [30].

Gene expression during endochondral bone formation is regulated by a series of chondrogenic and osteogenic inductive cell signaling, proliferation, and differentiation events at the undifferentiated mesenchymal stem/progenitor cell stage involving extracellular matrix remodeling, cartilage deposition and vasculogenesis, mineralization ossification, and subsequent replacement with bone [15, 24]. A cohort of gene transcripts and key osteogenic-related proteins (*RUNX-2, OCN, NOG, SP-1*) were identified that correlate to the early histological response and development of ectopic bone. *RUNX-2* is the early master regulator of osteogenic/osteoblast differentiation [16], and *OCN* is a prime marker of bone development produced by osteoblasts [18]. *SP-1* is a neuroinflammatory peptide that has been reported to promote the mobilization, proliferation, and osteogenic differentiation of mesenchymal stem cells at sensory nerve structures [25, 26]. We showed here that the expression of *SP-1* is induced systemically in our trauma-induced model, providing a connection between blast injury and increased formation of ectopic bone. Consistent with previous studies examining endochondral bone development and early ossification, we observed that HO is coupled with an early increase in the expression of transcripts necessary for synthesis of a cartilaginous matrix (*COL1α1*), bone and osteoblast mineralization (*RUNX-2, OCN, PHEX,* and *POU5F1*), tissue remodeling (*MMP9, CSF3, FGF-10,* and *HAS1*), and inflammatory cytokines (*IL-6, IL1β, CCL2, CCL3,* and *CXCL5*) within the first 14 days postinjury [14].

Recently, Peterson et al. [19] demonstrated in a murine Achilles tenotomy plus partial-thickness dorsum burn injury model that injured mice develop endochondral ectopic bone and functional joint contractures through BMP-mediated canonical small "mothers against" decapentaplegic (SMAD) signaling. Moreover, they report that these orthopaedic disease processes can be attenuated/modulated by targeting adenosine triphosphate (ATP) hydrolysis and

SMAD1/5/8 phosphorylation at the burn site using apyrase [19]. Interestingly, it has been reported that focused extracorporeal shockwave therapy (ESWT; low-density shockwaves administered orders of magnitude below blast overpressure conditions used in the study) has been shown to induce osteogenic differentiation of marrow-derived mesenchymal stem cells through ATP release and downstream transcriptional signaling events resulting in activation of P2X7 receptors [28]. Consistent with the finding of Peterson et al., removal of ATP using apyrase inhibited ESWT-induced osteogenic differentiation. ESWT has been used in treatment of bone and soft tissue disorders and shown to stimulate soft tissue expression of osteogenic factors (BMPs, OC, OPN, TGFβ1) but also angiogenic factors (VEGF, FGF) [27, 32]. Therefore, it is not surprising that many of the genes induced in the musculature of the injured limb show parallel, albeit reduced, levels of change in the contralateral limb.

In this study, we defined the histologic time course and pertinent molecular signaling patterns in the early stages of HO development using a rat model of combat-related HO that incorporates the critical elements associated with combat injury. Based on these findings, we propose that the initiation of prophylactic therapy targeted at inhibiting the synthesis of ectopic cartilage should start soon after injury in the rat to avoid any adverse effects on physiologic early wound healing processes such as tissue revascularization and granulation tissue development. The ability to correlate molecular events with histologic and morphologic changes will help researchers and clinicians to understand the HO process. In addition, ascertaining how applicable the findings are to the wound healing process in humans will be important in formulating therapeutic interventions that target early chondrogenic, angiogenic, and osteogenic signaling components of ectopic bone development.

**Acknowledgments** We thank Dana M. Golden and Allison M. Tomasino for assistance with animal care, sample collections, and technical expertise; Dr Matthew Wagner for his statistical analysis; and LTC Cary L. Hannold for his detailed histological review of specimens.

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# Preventative Therapeutics for Heterotopic Ossification

Log Number: OR120056P1
Award Number: W81XWH-13-2-0077

**PI:** CDR Jonathan Forsberg, MD **Org:** The Geneva Foundation **Award Amount:** $317,117.00

## Study Aims

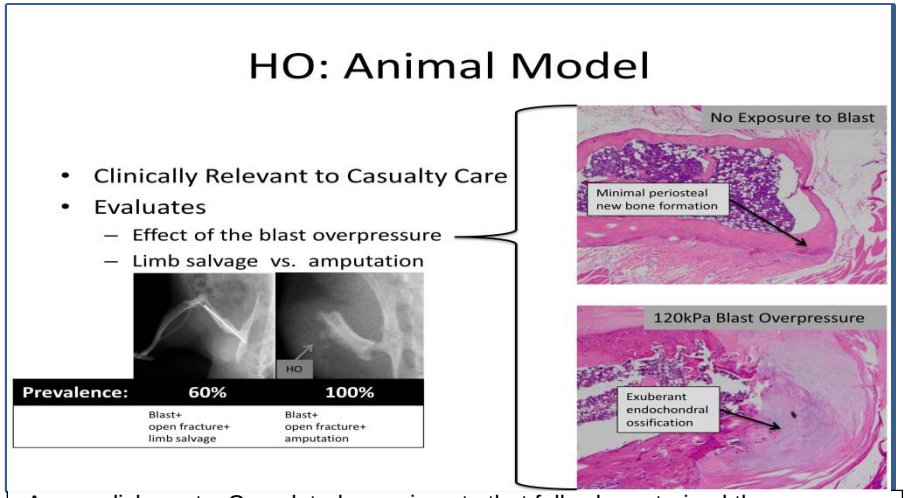
To determine whether the retinoid agonists block blast- and combat-related HO.

1. Implement the rat blast-injury model to include bacterial infection
2. Test drug effectiveness, regimens and systemic versus local deliver
3. Analyze wound healing and muscle repair

## Approach

We are using a modified rat model of blast-related extremity trauma that reproduces the systemic inflammatory and pathogenic state seen in wounded service members, including HO and bacterial infection.

## HO: Animal Model




Accomplishments: Completed experiments that fully characterized the experimental drug's effectiveness, treatment timing (scheduling) and potential side affects as a prophylactic measure for inhibiting HO development in our model of blast-induced extremity injury.

## Timeline and Cost

| Activities / CY | 13 | 14 | 15 | 16 |
|---|---|---|---|---|
| Implement model | | | | |
| Test drug effectiveness | | | | |
| Analyze wound healing | | | | |
| Assessment of bioburden on HO | | | | |
| **Estimated Budget ($K)** | **$37,607** | **$133,554** | **$83,136** | **$62,819** |

**Updated:** 20 October 2015

**Goals/Milestones**

**CY13 Goal** – Implement Model

- ❑ Anatomical samples will be HO tissue masses and limb long bones in limb-salvage group

**CY14 Goals** – Drug Effectiveness Testing

- ❑ Anatomical samples will be HO tissue masses and limb long bones in limb-salvage group

**CY15 Goal** – Wound and Muscle Repair Analysis

- ❑ Analyze samples

**Comments/Challenges/Issues/Concerns**

- Aims and milestones achieved in accordance to plans.

**Budget Expenditure to Date**

Actual Expenditure: $148,640.22